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DETERMINANTS OF *LISTERIA MONOCYTOGENES* STRESS RESPONSES

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ACADEMIC DISSERTATION

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ABSTRACT

Listeria monocytogenes is a remarkable bacterium, as it is able to shift from a capable environmental saprophyte into a severe intracellular pathogen. As a strictly foodborne pathogen, *L. monocytogenes* poses a notable risk, particularly to those consumers among the risk groups for whom invasive listeriosis is potentially fatal. Furthermore, modern consumption habits and increasingly favoured ready-to-eat foods, often consumed without proper heating, increase the risk of acquiring the foodborne disease. The aim of this study was to investigate the genetic mechanisms conferring wide-ranging stress tolerance in *L. monocytogenes*.

Two-component systems, comprising a sensor histidine kinase and a cognate response regulator, aid bacteria in sensing and adapting to changes in both surrounding environmental as well as intracellular conditions. The histidine kinases, in particular, have lacked comprehensive studies on their roles in the stress tolerance of *L. monocytogenes*. Thus, histidine kinases were studied by expressional analyses under cold conditions and by mutationally disrupting each histidine kinase-encoding gene in a parental model strain, *L. monocytogenes* EGD-e. The modified strains were individually challenged at high (42.5 °C) and low (3.0 °C) temperatures, high (9.4) and low (5.6) pH levels, and high salt (6% NaCl), ethanol (3.5 vol%) and hydrogen peroxide (5 mM) concentrations. Expressional studies and growth experiments on genetically modified strains proved *lisK* and *yycG* to respectively play central roles in the acclimation and immediate growth of *L. monocytogenes* at low temperatures. The most substantial increase in gene expression under cold conditions was that of the chemotaxis gene *cheY* with 236-fold upregulation at 3 °C. The disrupted Δ*liaS* strain displayed impaired growth in response to all the other stresses, particularly at a high temperature and under osmotic stress. These studies demonstrated the prominent importance of the histidine kinase-encoding genes *yycG* and *lisK* to cold tolerance and *liaS*, with roles in the growth of *L. monocytogenes* under multiple stresses.

To shed light on the accessory genetic mechanisms that cause large strain variation in *L. monocytogenes* in withstanding heat treatments, heat resistance-conferring traits were further investigated by means of whole-genome sequencing. Comparing the complete genomes of heat-resistant *L. monocytogenes* AT3E and -sensitive AL4E strains revealed the heat-resistant strain to harbour a novel 58-kb plasmid, pLM58, which was absent in the sensitive strain. Furthermore, curing of the plasmid in AT3E produced a marked decrease in heat resistance from virtually no reduction to a 1.1 cfu/ml $\log_{10}$ reduction at 55.0 °C. In pLM58, a 2,155-bp open reading frame annotated as an ATP-dependent ClpL protease-encoding gene was identified. Conjugation of the coding sequence and the putative promoter of the *clpL*

gene into a natively heat-sensitive *L. monocytogenes* 10403S strain, in turn, enhanced the survival of the strain from a 1.2 cfu/ml $\log_{10}$ reduction to a 0.4 cfu/ml $\log_{10}$ reduction in heat challenge at 55.0 °C. In this study, we presented the first evidence of plasmid-mediated heat resistance in *L. monocytogenes* and identified the protease ClpL to be a novel plasmid-borne heat-resistance mediator.

The emerging resistance of *L. monocytogenes* to benzalkonium chloride, a quaternary ammonium compound widely used as a detergent in food-processing facilities, is a significant concern for food safety and public health. The resistance of 392 *L. monocytogenes* isolates from Finland (n = 197) and Switzerland (n = 195) to benzalkonium chloride was assessed. A minimal inhibitory concentration of 20 μg/ml was defined. Altogether, 11.5% of the strains proved to be resistant to benzalkonium chloride. Serotype 1/2c harboured the highest prevalence, 32.4% (11/34), of benzalkonium chloride-resistant strains, while in total, most of the resistant strains belonged to serotype 1/2a. Altogether, 68.9% of the resistant strains harboured at least one of the efflux pump system-encoding genes, *bcrABC*, *emrE* or *qacH*, known to confer benzalkonium chloride resistance in *L. monocytogenes*. We found resistant strains with partially or completely efflux pump-dependent benzalkonium chloride resistance, with the exception of the known resistance-mediating efflux pumps, suggesting the existence of other resistance-contributing efflux pump systems. The lacking of known efflux pump system-encoding genes in addition to efflux pump-independent benzalkonium chloride resistance, in turn, indicates the contribution of completely novel benzalkonium chloride resistance mechanisms.

The aim of these studies was to shed light on the genes contributing to the versatile stress tolerance abilities and strain variation of the severe foodborne pathogen, *L. monocytogenes*. Knowledge of such traits may aid in developing targeted strategies and measures to identify and control the contamination and risks caused, in particular, by stress-tolerant *L. monocytogenes* strains.

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CONTENTS

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications, referred to in the text by their Roman numerals:

I. Pöntinen, A., Markkula, A., Lindström, M. & Korkeala, H. (2015) Two-component-system histidine kinases involved in growth of *Listeria monocytogenes* EGD-e at low temperatures. *Appl Environ Microbiol* 81: 3994-4004.

II. Pöntinen, A., Lindström, M., Skurnik, M. & Korkeala, H. (2017) Screening of the two-component-system histidine kinases of *Listeria monocytogenes* EGD-e. LiaS is needed for growth under heat, acid, alkali, osmotic, ethanol and oxidative stresses. *Food Microbiol* 65: 36-43.

III. Pöntinen, A., Aalto-Araneda, M., Lindström, M. & Korkeala, H. (2017) Heat resistance mediated by pLM58 plasmid-borne ClpL in *Listeria monocytogenes*. *MSphere* 2: e00364-17.

IV. Meier, A.B., Guldimann, C., Markkula, A., Pöntinen, A., Korkeala, H. & Tasara, T. (2017) Comparative phenotypic and genotypic analysis of Swiss and Finnish *Listeria monocytogenes* isolates with respect to benzalkonium chloride resistance. *Front Microbiol* 8: 397.

ABBREVIATIONS

A	Adenine
ADI	Arginine deiminase
AFLP	Amplified fragment length polymorphism
Asp	Aspartate
ATP	Adenosine triphosphate
AUC	Area under the curve
a_w	Water activity
BC	Benzalkonium chloride
BC^r	Benzalkonium chloride-resistant
BHI	Brain–heart infusion
bp	Base pair
C	Cytosine
Cap	Cold acclimation protein
CC	Clonal complex
cDNA	Complementary DNA
CDS	Coding sequence
cfu	Colony forming unit
Csp	Cold shock protein
DNA	Deoxyribonucleic acid
EC	Epidemic clone
ET	Electrophoretic type
FA	Fatty acid
G	Guanine
GABA	γ-aminobutyrate
GDA	Glutamate decarboxylase
GWAS	Genome-wide association studies
HGT	Horizontal gene transfer
His	Histidine
HK	Histidine kinase
HPt	His-containing phosphotransfer protein
Hsp	Heat shock protein
ICE	Integrative conjugative element
Inc	Incompatibility group
LB	Luria-Bertani (lysogeny broth)
LD	Linkage disequilibrium
LIPI	*Listeria* pathogenicity island
LLS	Listeriolysin S
MGE	Mobile genetic element
MH	Mueller-Hinton
MIC	Minimal inhibitory concentration
MLEE	Multilocus enzyme electrophoresis

MLST	Multilocus sequence typing
MLVA	Multiple-locus variable-number tandem repeat analysis
Mpf	Mating pair formation
mRNA	Messenger ribonucleic acid
NGS	Next-generation sequencing
OD_{600}	Optical density at 600 nm
ORF	Open reading frame
PCA	Plate count agar
PCR	Polymerase chain reaction
PFGE	Pulsed-field gel electrophoresis
QAC	Quaternary ammonium compound
RAPD	Randomly amplified polymorphic DNA
REA	Restriction endonuclease analysis
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RR	Response regulator
rRNA	Ribosomal ribonucleic acid
RTE	Ready-to-eat
RT-qPCR	Real-time quantitative reverse transcription-PCR
Sap	Salt acclimation protein
SD	Standard deviation
SMRT	Single-molecule real-time
SNP	Single nucleotide polymorphism
SOE	Splicing-by-overlap extension
SSI	Stress survival islet
Ssp	Salt shock protein
ST	Sequence type
T	Thymine
T4SS	Type IV secretion system
TCS	Two-component signal transduction system
TS	Tryptic soy
WGS	Whole-genome sequencing

1 INTRODUCTION

The first plausible discovery of *Listeria monocytogenes* dates back to 1911 when the Swedish veterinarian Gustav Hülphers described a bacterium isolated from the necrotic liver tissue of a rabbit and, accordingly, named his discovery *Bacillus hepatis* (Hülphers, 1911). However, the isolate itself was not preserved, and it was only after the discovery by Murray *et al.* (1926) that a detailed description of the bacterium was established, often designated the first report on *L. monocytogenes*. The bacterium was, at that time, named *Bacterium monocytogenes* due to it causing a characteristic proliferation of monocytes (Murray *et al.*, 1926). In 1927, the bacterium was newly named as *Listerella hepatolytica* (Pirie, 1927), and in 1940 it finally received its present name, *L. monocytogenes* (Pirie, 1940), in honour of Lord Lister, the *primus motor* of antisepsis and hygienic surgical measures.

Although *L. monocytogenes* was isolated from a human meningitis patient as early as in 1918 (Dumont and Cotoni, 1921), the first confirmed human listeriosis cases were reported a decade later (Nyfeldt, 1929). The foodborne nature of listeriosis was only explicitly recognised after distinct outbreaks in the 1980s (Schlech *et al.*, 1983, Fleming *et al.*, 1985, Linnan *et al.*, 1988), although the association between foodstuffs and listeriosis was already conjectured in the 1950s and after (Potel, 1953, Gray, 1963). Since then, foodborne listeriosis outbreaks have unfortunately become commonplace, and as consumption habits evolve to favour ready-to-eat foods without proper heating, *L. monocytogenes* continues to be a notable food safety hazard.

Besides being a foodborne pathogen, *L. monocytogenes* dwells ubiquitously in the environment, from soil and vegetation to water supplies and cultivated land. Indeed, it is characteristically able to shift from being an environmental saprophyte to an intracellular pathogen (Chaturongakul *et al.*, 2008, Cossart, 2011). Its versatile ability to endure and overcome stressful conditions plays a fundamental role in the transmission processes of the bacterium from the environment to the vehicle foods and feed and, ultimately, into the animal or human host. Notably, *L. monocytogenes* copes well with many of the stressors, such as refrigeration and acidic and osmotic conditions, that are frequently applied to limit the number of bacteria in food-processing environments and foodstuffs (Bucur *et al.*, 2018). Thus, identifying the genetic mechanisms conferring this agile phenotypic stress tolerance is a pivotal step in understanding the survival strategies of the bacterium and, moreover, in controlling *L. monocytogenes* contamination along the food chain.

Genetic research in bacteriology has undergone marked changes in the recent years, as study methods have evolved from traditional molecular techniques to the emergence of novel whole-genome sequencing-based

approaches (Read and Massey, 2014). Next-generation sequencing methods have facilitated the study of accessory genetic mechanisms and their role in conferring stress resistance between bacterial strains and populations. Both genomic and phenotypic comparisons in the model *L. monocytogenes* strains have demonstrated how strain variability may affect the observations that we make based on the *in vitro* studies performed on these strains and lead to discrepant results (Bécavin *et al.*, 2014). High-throughput and genome-wide methods allow us to more efficiently acknowledge and take into account this strain variability and thereby avoid possible misconceptions in the interpretation of research findings due to naturally occurring differences between strains (Brul *et al.*, 2012, Bécavin *et al.*, 2014).

Expressional analyses and mutational studies have enabled the role of many genetic mechanisms to be ascertained in the stress tolerance of *L. monocytogenes*, such as stress shock proteins and membrane transport systems (Gandhi and Chikindas, 2007). Whole-genome sequencing, in turn, has provided further new tools to process hundreds to thousands of strains simultaneously and to identify even larger sets of culprit stress tolerance and virulence genes (Read and Massey, 2014, Kachroo *et al.*, 2019). However, many proteins encoded by unknown or putative genes still remain. Thus, the overall picture of the genetics underlying *L. monocytogenes* stress tolerance in foods and food-processing environments is anything but complete and warrants further investigations into the stress responses of the bacterium.

2 REVIEW OF THE LITERATURE

2.1 *Listeria* spp. and *Listeria monocytogenes*

2.1.1 *Listeria* spp.

Listeria spp. belong to the family *Listeriaceae* in the order *Bacillales* and class *Bacilli* of the phylum *Firmicutes* (McLauchlin and Rees, 2015). Currently, together with *Listeria monocytogenes* (*sensu stricto*) (Murray *et al.*, 1926, Pirie, 1940), the validated *Listeria* species (McLauchlin and Rees, 2015) include *L. grayi* (Larsen and Seeliger, 1966, Rocourt *et al.*, 1992), *L. innocua* (Seeliger, 1984), *L. ivanovii* (Seeliger *et al.*, 1984), *L. seeligeri* and *L. welshimeri* (Rocourt and Grimont, 1983). In addition, *L. marthii* (Graves *et al.*, 2010) and the phylogenetically more divergent *Listeria* species *L. rocourtiae* (Leclercq *et al.*, 2010), *L. fleischmannii* (Bertsch *et al.*, 2013), and *L. weihenstephanensis* (Lang Halter *et al.*, 2013) have been described.

In recent years, several novel species have been isolated, including *L. aquatica* sp. nov., *L. cornellensis* sp. nov., *L. floridensis* sp. nov., *L. grandensis* sp. nov., *L. riparia* sp. nov. (den Bakker *et al.*, 2014), *L. booriae* sp. nov. and *L. newyorkensis* sp. nov. (Weller *et al.*, 2015). Additionally, subspecies *L. ivanovii* subsp. *ivanovii* and subsp. *londoniensis* have been designated within *L. ivanovii* (Boerlin *et al.*, 1992), as well as subspecies *L. fleischmannii* subsp. *fleischmannii* and subsp. *coloradonensis* within *L. fleischmannii* (den Bakker *et al.*, 2013). The formerly described *L. murrayi* (Welshimer and Meredith, 1971) has since been designated as a single species within *L. grayi* (Rocourt *et al.*, 1992).

2.1.2 Characteristics of *L. monocytogenes*

L. monocytogenes is a Gram-positive, facultative anaerobic, short rod (0.4–0.5 × 1–2 μm). It forms peritrichous flagella and manifests a characteristic tumbling motility at <30 °C. *L. monocytogenes* produces neither spores nor capsules, but it may develop filaments of ≥6 μm in length (Gray and Killinger, 1966, McLauchlin and Rees, 2015). It is β-haemolytic, catalase-positive and oxidase-negative, and typically ferments rhamnose but not xylose (Roberts *et al.*, 2006, McLauchlin and Rees, 2015). *L. monocytogenes* harbours a low average DNA G+C content (mol%) of 39% (Glaser *et al.*, 2001), varying from 36% to 42.5% (McLauchlin and Rees, 2015).

L. monocytogenes isolates form a structured population, currently divided into four evolutionary lineages (Orsi *et al.*, 2011, Tsai *et al.*, 2011) (Table 1). The majority of *L. monocytogenes* strains belong to lineages I and II (Doumith *et al.*, 2004), first described by Piffaretti *et al.* (1989). These

lineages are clearly distinguishable in regard to common sources and genetic characteristics, while lineages III and IV are comprised of more diverse strains.

A clonal genetic structure has been proposed for *L. monocytogenes*, supported by virulence gene allelic analysis, ribotyping and extensive linkage disequilibrium (LD) within the virulence genes and certain lineage I and II electrophoretic types (ETs) (Piffaretti *et al.*, 1989, Wiedmann *et al.*, 1997). However, based on the diversity of housekeeping, virulence and stress response genes, Nightingale *et al.* (2005b) later reported that while lineage I is indeed highly clonal, isolates of lineage II display greater genetic diversity. Furthermore, through genome-wide analyses, recombination has been determined to occur more commonly in lineage II than lineage I isolates (Orsi *et al.*, 2008). A high recombination rate could explain the diverse isolation sources of lineage II when compared to the seemingly more host-adapted lineage I (Orsi *et al.*, 2008). Besides atypical 4b serotypes (Roberts *et al.*, 2006, Orsi *et al.*, 2011), rhamnose-negative serotype 4c and 4a strains have been reported in lineages III and IV, respectively (Liu *et al.*, 2006).

Table 1. Summary of *L. monocytogenes* lineages and serotypes.

Lineage	Serotypes	Genetic characteristics	Sources	References
I	1/2b, 3b, 3c, 4b, 4d, 4e	Clonal	Various sources, human isolates overrepresented	(Piffaretti *et al.*, 1989, Bibb *et al.*, 1990, Nadon *et al.*, 2001, Ward *et al.*, 2004)
II	1/2a, 1/2c, 3a, 3c	Recombinant	Various sources, food-related and environmental isolates, sporadic human isolates	(Piffaretti *et al.*, 1989, Bibb *et al.*, 1990, Ward *et al.*, 2004)
III	4a, 4b (atypical), 4c	Diverse isolates, moderate recombination rates	Predominantly animal origin, rare human and food isolates	(Rasmussen *et al.*, 1995, Nadon *et al.*, 2001, Ward *et al.*, 2004, Nightingale *et al.*, 2005b, Roberts *et al.*, 2006)
IV	4a, 4b (atypical), 4c, 7	Few isolates to date	Few animal, human, and food isolates	(Roberts *et al.*, 2006) (IIIB); (Liu *et al.*, 2006, Ward *et al.*, 2008)

Typing

L. monocytogenes strains are phenotypically categorised into 13 serotypes, when also including serotype 4ab, based on cell surface heat-stable somatic (O) and heat-labile flagellar (H) antigens (Seeliger and Höhne, 1979). In addition to serotyping, other conventional phenotypic methods, such as multilocus enzyme electrophoresis (MLEE) (Bibb *et al.*, 1989, Piffaretti *et al.*,

1989) and phage typing (Audurier *et al.*, 1979, Rocourt *et al.*, 1985), can be applied to type *L. monocytogenes* isolates. More novel, genotypic subtyping methods include amplified fragment length polymorphism (AFLP) (Vos *et al.*, 1995, Keto-Timonen *et al.*, 2003), pulsed-field gel electrophoresis (PFGE) (Ojeniyi *et al.*, 1991, Ojeniyi *et al.*, 1996, Katzav *et al.*, 2006), PCR-based randomly amplified polymorphic DNA (RAPD) (Mazurier and Wernars, 1992, Lawrence *et al.*, 1993), restriction endonuclease analysis (REA) (Nocera *et al.*, 1990, Ericsson *et al.*, 1993), PCR-restriction fragment length polymorphism (RFLP) (Vines *et al.*, 1992), ribotyping (Graves *et al.*, 1991, Graves *et al.*, 1994) and DNA sequencing-based subtyping methods, such as multilocus sequence typing (MLST) (Salcedo *et al.*, 2003, Haase *et al.*, 2014, Moura *et al.*, 2016), multiple-locus variable-number tandem repeat analysis (MLVA) (Keim *et al.*, 2000, Murphy *et al.*, 2007, Lindstedt *et al.*, 2008) and single nucleotide polymorphism (SNP) typing (Nelson *et al.*, 2004). Epidemic clones (ECs) consist of phenotypically and genotypically closely related epidemic-associated *L. monocytogenes* isolates, even though isolates of the same epidemic clone might cause outbreaks geographically or temporally distant from each other (Piffaretti *et al.*, 1989, Kathariou, 2002). The first sequenced *L. monocytogenes* genome was that of the EGD-e strain (Glaser *et al.*, 2001). Subsequently, whole-genome sequencing (WGS)-based approaches have become increasingly common typing methods in bacteriology (Salipante *et al.*, 2015) and have facilitated large epidemiological outbreak investigations and surveillance of infectious diseases (Kwong *et al.*, 2016, Revez *et al.*, 2017, Schjørring *et al.*, 2017, Allam *et al.*, 2018).

2.1.3 *L. monocytogenes* contamination

L. monocytogenes is a ubiquitous saprophyte in the environment (Weis and Seeliger, 1975). It can be found in soil and vegetation, in water supplies and sewage, on feeding grounds, in animal and human faeces and in poorly prepared fodder (Weis and Seeliger, 1975, Husu *et al.*, 1990a, Renterghem *et al.*, 1991, MacGowan *et al.*, 1994, Unnerstad *et al.*, 2000, Pauly and Tham, 2003, Lyautey *et al.*, 2007a, Lyautey *et al.*, 2007b).

Besides being a saprophyte dwelling in the environment, *L. monocytogenes* has been isolated from numerous raw foods, with the highest prevalences reported in poultry (Miettinen *et al.*, 2001, Rørvik *et al.*, 2003) and a somewhat more seldom occurrence in other raw meats and fish (Autio *et al.*, 1999, Autio *et al.*, 2000, Markkula *et al.*, 2005). Although the total number of bacteria and *Listeria* spp. is generally fairly low in Finnish bulk tank milk (Ruusunen *et al.*, 2013), *L. monocytogenes* has also been isolated in raw milk samples (Husu, 1990, Ruusunen *et al.*, 2013, Castro *et al.*, 2017). Epidemiological studies have implied faecal contamination to be a putative cause of the frequent dissemination of *L. monocytogenes* in tank milk and raw meat products (Skovgaard and Nørrung, 1989, Husu, 1990,

Husu *et al.*, 1990b). A longitudinal study employing PFGE further indicated that *L. monocytogenes* contamination in tank milk may derive from udders or milking equipment (Castro *et al.*, 2018). *L. monocytogenes* has also been isolated in food-processing plants, where certain subtypes have persisted over long periods of months to several years (Miettinen *et al.*, 1999a, Vogel *et al.*, 2001, Hoffman *et al.*, 2003, Lundén *et al.*, 2003b, Keto-Timonen *et al.*, 2007, Bērziņš *et al.*, 2010, Almeida *et al.*, 2013). *L. monocytogenes* is thus potentially a highly challenging contaminant in food-processing environments.

The initial occurrence of *L. monocytogenes* in slaughterhouses and other food-processing premises in the production chain has frequently been indicated to derive from raw materials (Autio *et al.*, 2000, Berrang *et al.*, 2002, Autio *et al.*, 2004, Markkula *et al.*, 2005). However, tracing *L. monocytogenes* strains with phenotypic or genetic molecular typing methods has revealed contamination of the final products originating from sources other than raw material (Rørvik *et al.*, 1995, Autio *et al.*, 1999) or not exclusively incriminating the raw material as a contamination source (Norton *et al.*, 2001, Vogel *et al.*, 2001). Continuous listerial contamination in a food-processing facility may result from persistent strains dwelling in the processing environment rather than repeatedly deriving from incoming raw materials (Hoffman *et al.*, 2003). Recontamination of food products in the processing line after heating is a frequent source for the occurrence of *L. monocytogenes* in processed foods (Tompkin, 2002, Reij *et al.*, 2004). This is of importance particularly concerning ready-to-eat (RTE) foods, which are often consumed without heating.

2.2 Listeriosis

Of *Listeria* spp., *L. monocytogenes* and *L. ivanovii* are considered pathogenic (Nyfeldt, 1929, Ivanov, 1962, Vazquez-Boland *et al.*, 2001). *L. monocytogenes* is the predominant cause of listeriosis in both humans and animals, while *L. ivanovii* mainly leads to illness in ruminants (Ramage *et al.*, 1999, Vazquez-Boland *et al.*, 2001). Rare sporadic human cases caused by *L. ivanovii*, *L. innocua* and *L. grayi* have been reported, with predisposing factors and symptoms similar to those associated with *L. monocytogenes* (Cummins *et al.*, 1994, Lessing *et al.*, 1994, Perrin *et al.*, 2003, Snapir *et al.*, 2006, Rapose *et al.*, 2008, Guillet *et al.*, 2010).

2.2.1 Human listeriosis

The incidence of reported human listeriosis cases in EU/EEA countries is approximately 0.6 and in the USA 0.3 sporadic cases per 100 000 population annually (Silk *et al.*, 2012, CDC, 2013, ECDC, 2016). Even though invasive listeriosis is a fairly rare condition, it is a severe illness with an average case-

fatality rate of 20% to 30% (Fleming *et al.*, 1985, Mylonakis *et al.*, 1998, Mead *et al.*, 1999, de Valk *et al.*, 2005, CDC, 2013, Crim *et al.*, 2014). In patients suffering from listerial meningitis, mortality of up to 36% has been reported (Koopmans *et al.*, 2013).

Listeriosis is likely to manifest as sporadic cases, but outbreaks have also been reported (Fleming *et al.*, 1985, Linnan *et al.*, 1988, McLauchlin, 1996, Lyytikäinen *et al.*, 2000, Swaminathan and Gerner-Smidt, 2007, Angelo *et al.*, 2017, Schjørring *et al.*, 2017, Allam *et al.*, 2018). Most (99%) of the listeriosis cases are foodborne (Schlech *et al.*, 1983, Pinner *et al.*, 1992, Mead *et al.*, 1999, Swaminathan and Gerner-Smidt, 2007). However, aberrant transmission from animals to humans has been reported, mostly as localised infections among farmers and veterinarians (McLauchlin and Low, 1994, Regan *et al.*, 2005). As most listeriosis cases are sporadic and the incidence is low, and due to the long incubation period of invasive listeriosis and the subsequent delay in diagnosis, the vehicle foods are often challenging to trace. However, a high risk of acquiring listeriosis is frequently attributable to RTE foods with prolonged shelf lives and consumed without proper heating (Schwartz *et al.*, 1988, Pinner *et al.*, 1992, Cartwright *et al.*, 2013, Lopez-Valladares *et al.*, 2018). In recent years, outbreaks have been linked to a varied range of such foods, including caramel apples (Angelo *et al.*, 2017), ice cream products (Chen *et al.*, 2017) and cold-smoked salmon (Schjørring *et al.*, 2017).

L. monocytogenes is a facultative intracellular pathogen (Cossart, 2011, Radoshevich and Cossart, 2018). The virulence genes essential for the intracellular lifecycle of the bacterium are clustered in the chromosomal *Listeria* pathogenicity islands (LIPIs) (Vázquez-Boland *et al.*, 2001). *L. monocytogenes* enters the host via the gastrointestinal tract and, while in the intestine, may secrete the bacteriocin compound listeriolysin S (LLS) to restrict the growth of competing commensal bacteria in the host (Cotter *et al.*, 2008, Quereda *et al.*, 2016). *L. monocytogenes* is able to invade the intestinal barrier through junctions between mucus-producing goblet cells and enterocytes, via epithelial M cells in the Peyer's patches, or through the tip of the intestinal villi where apoptotic epithelial cells extrude into the intestinal lumen (Jensen *et al.*, 1998, Nikitas *et al.*, 2011, Ribet and Cossart, 2015, Radoshevich and Cossart, 2018). *L. monocytogenes* is able to infect both macrophages and nonphagocytic cells and, once intracellular, can spread while evading the hostile extracellular environment (Berche *et al.*, 1988, Cossart, 2011, Pizarro-Cerdá *et al.*, 2012, Jones *et al.*, 2015). After penetrating the intestinal barrier, *L. monocytogenes* transfers to the spleen and liver, where it is eliminated by the T-cell-mediated immune system and Kupffer cells (Ebe *et al.*, 1999, Gregory and Liu, 2000, Pope *et al.*, 2001, Shi *et al.*, 2010, Blériot *et al.*, 2015). However, in hosts with depressed cell-mediated immunity, *L. monocytogenes* may spread into the circulatory system and cause bacteraemia. Furthermore, it may cross the blood–brain barrier or during pregnancy the placenta and, consequently, cause the

symptoms of invasive listeriosis (Mylonakis *et al.*, 1998, Mylonakis *et al.*, 2002, Bakardjiev *et al.*, 2006, Disson and Lecuit, 2012).

High-risk groups for invasive listeriosis include neonates, the elderly and individuals immunocompromised due to comorbid malignant diseases, autoimmune disorders, organ transplantation, immunosuppressive medication or pregnancy (Goulet and Marchetti, 1996, Mylonakis *et al.*, 1998, Goulet *et al.*, 2012, Silk *et al.*, 2012). Invasive listeriosis manifests most commonly as bacteraemia or septicaemia, meningitis and meningoencephalitis. Less frequently, pneumonia, peritonitis and other focal infections may occur (Ericsson *et al.*, 1997, López-Prieto *et al.*, 2000, Schlech, 2000, Swaminathan and Gerner-Smidt, 2007, Aubin *et al.*, 2016). During pregnancy, infection may lead to abortion or neonatal listeriosis, while pregnant women frequently display non-specific symptoms resembling influenza (Evans *et al.*, 1985, Mylonakis *et al.*, 2002, Vázquez-Boland *et al.*, 2017). Early-onset neonatal listeriosis derives from intrauterine infection. It can result in stillbirth or septicaemia and pneumonia in the infant within a few days after delivery. The rarer late-onset form is considered to derive from passage through the birth canal during labour. Meningitis, as the most common clinical presentation, occurs during a few weeks following delivery (Evans *et al.*, 1985, Mylonakis *et al.*, 2002, Sapuan *et al.*, 2017, Vázquez-Boland *et al.*, 2017).

In healthy individuals, *L. monocytogenes* may cause a noninvasive infection presenting as febrile gastroenteritis (Salamina *et al.*, 1996, Dalton *et al.*, 1997, Miettinen *et al.*, 1999b, Aureli *et al.*, 2000, Frye *et al.*, 2002, Ooi and Lorber, 2005, Jacks *et al.*, 2016). Sporadic cases of localised eye and cutaneous infections have also been described, which are frequently, but not exclusively, acquired through direct exposure of the site of infection instead of foodborne transmission and haematogenous dissemination (Betriu *et al.*, 2001, Regan *et al.*, 2005, Tay *et al.*, 2008, Godshall *et al.*, 2013).

Invasive listeriosis usually presents a prolonged incubation period of approximately 20 to 30 days, but this may vary widely from one up to 70 days (Linnan *et al.*, 1988, Goulet *et al.*, 2013). The minimum infectious dose of *L. monocytogenes* for either healthy or immunocompromised individuals is unclear, but the development of clinical illness appears to be dose-dependent (Ooi and Lorber, 2005). Prolonged daily intake may further increase the risk (Maijala *et al.*, 2001). In risk groups, *L. monocytogenes* levels of $>10^2$ cfu/g have caused invasive listeriosis (Ericsson *et al.*, 1997, Maijala *et al.*, 2001). In noninvasive listeriosis, the onset of symptoms is more rapid and usually takes place within a day or two after the ingestion of contaminated food, with a median of 18 to 31 hours (Salamina *et al.*, 1996, Dalton *et al.*, 1997, Miettinen *et al.*, 1999b, Aureli *et al.*, 2000, Frye *et al.*, 2002, Sim *et al.*, 2002, Carrique-Mas *et al.*, 2003). The infectious dose is usually markedly higher than that in invasive cases, approximately 10^5 to 10^9 cfu/g, although inocula down to 3×10^1 cfu/g have been detected in a potential vehicle food of noninvasive gastroenteritis (Dalton *et al.*, 1997,

Miettinen *et al.*, 1999b, Aureli *et al.*, 2000, Frye *et al.*, 2002, Sim *et al.*, 2002, Carrique-Mas *et al.*, 2003).

While *L. monocytogenes* strains isolated from food samples are most commonly serogroup 1/2 strains (Gilot *et al.*, 1996, Gianfranceschi *et al.*, 2003), the most common serotypes causing listeriosis are 4b, 1/2a and 1/2b (McLauchlin, 1990, Gilot *et al.*, 1996, Gianfranceschi *et al.*, 2003, Pontello *et al.*, 2012, Koopmans *et al.*, 2013, Prieto *et al.*, 2016): serotype 1/2a has been overrepresented in clinical cases in the Nordic countries (Lukinmaa *et al.*, 2003, Parihar *et al.*, 2008, Lopez-Valladares *et al.*, 2018), while 4b has been associated with high rates of hospitalisations and deaths particularly in North America (Pinner *et al.*, 1992, Cartwright *et al.*, 2013). Moreover, serotype 1/2a may gradually be outnumbering 4b as the most common serotype causing listeriosis worldwide, possibly due to its recurrent occurrence in increasingly favoured RTE foods (Lopez-Valladares *et al.*, 2018). However, based on putative associations between virulence and serotypes, McLauchlin (1990) suggested 4b to be more virulent than other serotypes. Ward *et al.* (2008) found by multilocus genotyping that mutations in *inlA*, leading to truncated InlA and decreased virulence, were exclusively harboured by serogroup 1/2 isolates, which may have explained the past overrepresentation of 4b isolates in listeriosis cases (Ward *et al.*, 2008). Notably, the bacteriocin LLS, beneficial for the invasion process inside host intestines, has only been recognised among a subset of lineage I strains (Cotter *et al.*, 2008, Quereda *et al.*, 2016), possibly further explaining the differences in virulence between lineages and serotypes. In the WGS era, putative virulence loci, such as the *Listeria* pathogenicity island LIPI-4, have been recognised in particularly virulent clinical clonal complexes (CC) among lineage I, which could explain their tropism for the placenta and central nervous system (Maury *et al.*, 2016, Radoshevich and Cossart, 2018).

2.2.2 Animal listeriosis

Among domestic animals, listeriosis mainly occurs in small ruminants and to a lesser extent in cattle, although nearly every domestic species is potentially susceptible to listeriosis (Nightingale *et al.*, 2004, Lecuit, 2007). Rarely, pathogenic *Listeria* spp. has been isolated in livestock and wildlife species such as swine, horses, fish and birds (Lecuit, 2007). Besides *L. monocytogenes*, *L. ivanovii* is also pathogenic in animals (Gouin *et al.*, 1994, Chand and Sadana, 1999). As in humans, a number of outbreaks along with sporadic cases have been reported in animals (Wilesmith and Gitter, 1986, Wiedmann *et al.*, 1996, Chand and Sadana, 1999, Lecuit, 2007).

The course of infection in animals is predominantly similar to that described in human listeriosis (Farber *et al.*, 1991, Lecuit, 2007). Clinical listeriosis in bovine, ovine and caprine livestock most often manifests as septicaemia, meningoencephalitis or, in gravid individuals, abortion (Wilesmith and Gitter, 1986, Campero *et al.*, 2002, Lecuit, 2007).

Septicaemic manifestation is usually seen in neonates. Rarely, ocular infections, mastitis and gastroenteritis have been reported (Lecuit, 2007). Clinically healthy animals may carry and shed the bacteria, and cattle in particular have been considered to transmit *L. monocytogenes* in the farm environment (Husu, 1990, Renterghem *et al.*, 1991, Nightingale *et al.*, 2004).

Predisposing factors for animal listeriosis include contaminated feed, deficient housing and management practices, and poor animal health. Similarly to human infections, the most common source of animal listeriosis is poorly prepared feed, with spoiled silage most often indicated as a culprit of transmission in farm animals (Wilesmith and Gitter, 1986, Husu *et al.*, 1990a, Vázquez-Boland *et al.*, 1992, Wiedmann *et al.*, 1996, Nightingale *et al.*, 2005a) Ruminants have also acquired infection through abrasions in the buccal mucosa followed by unilateral ascending neuritis along the trigeminal nerve (Braun *et al.*, 2002).

2.3 Plasmids in *Listeria* spp.

Plasmids, like other mobile genetic elements (MGEs), are capable of mediating genetic material between cells through horizontal gene transfer (HGT) (Frost *et al.*, 2005, Rankin *et al.*, 2011). HGT mechanisms include the uptake of free DNA from the environment via transformation, transduction conducted by bacteriophages, and conjugation, in which conjugative plasmids or integrative conjugative elements (ICEs) are transferred via a mating pair formation (Mpf) complex between two adjacent cells (Frost *et al.*, 2005, Thomas and Nielsen, 2005, Goessweiner-Mohr *et al.*, 2014). Natural competence, i.e. ability to uptake extracellular DNA via transformation, has not been detected in *L. monocytogenes* (Borezee *et al.*, 2000), while conjugation and transduction do occur.

The plasmid DNA transport machinery is encoded by *tra* operons harboured by the plasmid itself. For a complete conjugative apparatus of self-transmissible plasmids, the origin of transfer (*oriT*), relaxase, type IV coupling protein and the type IV secretion system (T4SS) are required. Mobilisable plasmids, in turn, utilise the T4SS of other co-resident conjugative plasmids in order to transmit via conjugation (Schröder and Lanka, 2005, Abajy *et al.*, 2007, Smillie *et al.*, 2010, Goessweiner-Mohr *et al.*, 2014).

Plasmids are most often circular, double-stranded DNA molecules, while some bacteria may harbour linear plasmids (Hayakawa *et al.*, 1979, Hinnebusch and Tilly, 1993). Plasmids control their copy number independently. They are self-replicating entities, with circular plasmids applying theta or rolling-circle replication (del Solar *et al.*, 1998, Slater *et al.*, 2008). Plasmids are classified into incompatibility groups (Inc) by replication control mechanisms and partitioning functions. Plasmids within

one Inc group cannot coexist in the same cell (Thomas and Smith, 1987, Frost *et al.*, 2005).

The presence of native plasmids in *Listeria* spp. was first described by Pérez-Díaz *et al.* (1982). Since then, a little over one-third of *L. monocytogenes* strains on average have been found to harbour plasmids, with isolation rates varying from 0% to 87% (Pérez-Díaz *et al.*, 1982, Flamm *et al.*, 1984, Fistrovici and Collins-Thompson, 1990, Lebrun *et al.*, 1992, Peterkin *et al.*, 1992, Dykes *et al.*, 1994, McLauchlin *et al.*, 1997, Margolles and de los Reyes-Gavilán, 1998, Fox *et al.*, 2016, Hingston *et al.*, 2017b). Infrequently, two nonidentical plasmids of similar or more divergent sizes have been found to coexist in a single *Listeria* spp. isolate (Kolstad *et al.*, 1991, Lebrun *et al.*, 1992, Dykes *et al.*, 1994, McLauchlin *et al.*, 1997, Hingston *et al.*, 2017b).

L. monocytogenes plasmids are more often associated with lineage II than lineage I strains (Lebrun *et al.*, 1992, McLauchlin *et al.*, 1997, Margolles and de los Reyes-Gavilán, 1998, Orsi *et al.*, 2011, Hingston *et al.*, 2017b). Within serotype 1/2a, plasmids have more often been isolated from food-related strains compared to clinical ones, while in serotype 1/2c, plasmids seem to be more abundant in clinical isolates (Lebrun *et al.*, 1992, McLauchlin *et al.*, 1997).

L. monocytogenes plasmids are mosaics but share many highly homologous fragments (Kolstad *et al.*, 1991, Canchaya *et al.*, 2010, Gilmour *et al.*, 2010). While a common replicon type has been recognised, suggesting a common ancestral origin, Kuenne *et al.* (2010) found that *Listeria* spp. plasmids can be divided into two distinct phylogenetic groups based on their *repA*-encoded replication initiation proteins. Of these, group 2 plasmids are larger and display more heterogeneity than group 1 (Kuenne *et al.*, 2010, Hingston *et al.*, 2017b). Comparative genetic analysis has revealed multiple phage defence systems to be found only among group 2 plasmids, suggesting a role for these plasmids in resisting phage infections (Kuenne *et al.*, 2010).

2.4 Two-component systems

Two-component signal transduction systems (TCSs) allow bacteria to rapidly sense and adapt to a varied range of stressors. The prototypical modular TCS consists of a periplasmic sensor histidine kinase (HK) and a cognate cytoplasmic response regulator (RR) (Fig. 1). HK protein harbours a variable N-terminal sensor (input) domain and a conserved C-terminal cytoplasmic kinase (transmitter) domain, linked by a transmembrane region with a variable number of segments spanning the cell membrane. Unlike membrane-bound HK sensors, some HKs are soluble cytoplasmic proteins, able to detect changes in the intracellular environment. The RR, in turn, is comprised of a conserved N-terminal regulatory (receiver) domain and a variable C-terminal effector (output) domain (Chang and Stewart, 1998,

Stock *et al.*, 2000). The variable domains within HK and RR respectively reflect the need to detect a vast number of specific stimuli and to generate an appropriate response by the cell (Gao and Stock, 2009).

The operation of TCSs is based on protein phosphorylation. In the direct phosphotransfer system, the sensor domain of the HK detects a stimulus, leading to ATP-dependent autophosphorylation at a specific histidine (His) residue, catalysed by the kinase domain. The cognate RR then catalyses the transfer of the phosphoryl group to its own aspartate (Asp) residue in the regulatory domain. Phosphorylation of the regulatory domain of the RR leads to activation of the effector domain and, finally, to an appropriate response to a particular stimulus. Predominantly, RRs function as DNA-binding transcription factors (West and Stock, 2001, Casino *et al.*, 2010). More complex phosphorelays may employ multiple HK and RR proteins, hybrid HKs with both His and Asp residues, and His-containing phosphotransfer proteins (HPt) that act as an intermediary in phosphoryl group transmission (Appleby *et al.*, 1996, West and Stock, 2001).

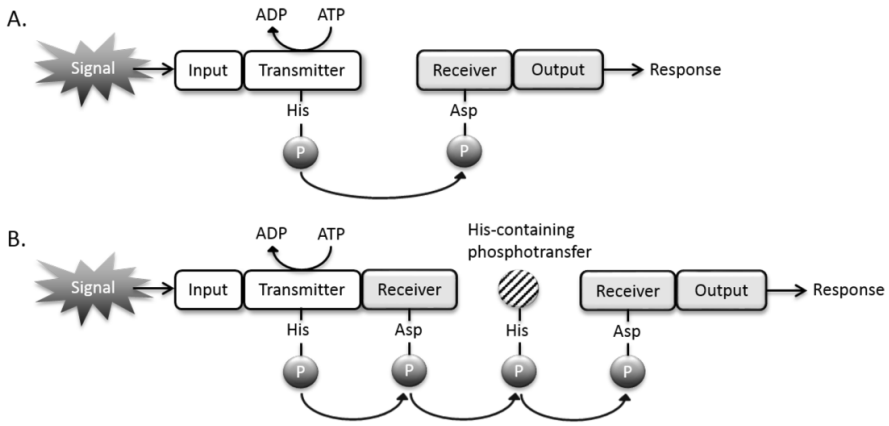

Figure 1 Schematic representation of a prototypical two-component system (A) and a phosphorelay with a hybrid histidine kinase and a His-containing phosphotransfer (B). Modified from (Mitrophanov and Groisman, 2008).

The RR phosphorylation level defines the extent of the output response. Dephosphorylation of RR may be catalysed by its own autophosphatase activity or by bifunctional HKs, limiting the active state (Lois *et al.*, 1993, Weiss *et al.*, 2002). HK autophosphorylation and phosphotransfer activity are also potential signal transfer control steps (Casino *et al.*, 2010).

Besides signalling between cognate HK and RR, signal transduction may take place between noncognate partners. In branched signalling pathways, one HK may phosphorylate multiple RRs (one-to-many) or several HKs one RR (many-to-one) (Laub and Goulian, 2007, Goulian, 2010). An example of

such a branched pathway is the chemotaxis machinery with HK CheA phosphorylating the RRs CheB and CheY (Kirby, 2009). In *Escherichia coli*, cross-talk between HK PmrB and its cognate RR PmrA, as well as noncognate QseB, augments polymyxin B resistance (Guckes *et al.*, 2017).

TCSs are abundant in eubacteria and have also been identified, although less frequently, in archaea and eucaryotes, while being absent in animals and humans (West and Stock, 2001, Wuichet *et al.*, 2010). An estimated 1% of the proteins encoded in eubacteria are comprised of TCSs (West and Stock 2001). However, the number of TCSs varies greatly between bacterial species. In *Synechochystis* sp., 80 open reading frames (ORFs) encode TCS proteins (Mizuno *et al.*, 1996), while the *Mycoplasma genitalium* genome harbours none (Mizuno, 1998). In the sequenced genome of *L. monocytogenes* EGD-e, 16 TCSs have been identified (Glaser *et al.*, 2001). Of these, 15 are complete signalling systems, while one, DegU, is an orphan RR lacking a cognate genetically linked HK (Williams *et al.*, 2005a).

2.5 Stress responses of *L. monocytogenes*

L. monocytogenes surmounts many diverse and harsh stress conditions both in the environment and inside host organisms. While it displays optimal growth at temperatures from 30 °C to 37 °C (McLauchlin and Rees, 2015), it is able to both survive and grow over a wide temperature range from <0 °C to 45 °C (Junttila *et al.*, 1988, Walker *et al.*, 1990, Hudson *et al.*, 1994, McLauchlin and Rees, 2015). In a vacuum, growth has been detected down to −1.5 °C (Hudson *et al.*, 1994). However, *L. monocytogenes* does not survive pasteurisation treatment at 60 °C for 30 minutes (McLauchlin and Rees, 2015) nor at 72 °C for 15 seconds (Bradshaw *et al.*, 1985). *L. monocytogenes* grows in a water activity down to 0.9 (Nolan *et al.*, 1992) and in salt concentrations of up to 10% (w/v) (Simon, 1956, McLauchlin and Rees, 2015). Combined with low temperature (5 ˚C), survival has been detected in 18% NaCl (Cole *et al.*, 1990). *L. monocytogenes* grows at a pH as high as 9.6 (Simon, 1956, Gray and Killinger, 1966). Under acidic conditions, growth has been reported at pH 4.4 (George *et al.*, 1988) and, combined with low temperature (5 °C), survival at a pH down to 4.2 has been observed (Cole *et al.*, 1990). *L. monocytogenes* is a facultative anaerobic bacterium and thus able to grow under both aerobic and anaerobic conditions (Jydegaard-Axelsen *et al.*, 2005, McLauchlin and Rees, 2015). Consequently, refrigeration, vacuum packaging and preservation measures relying on a high salt concentration or extreme pH values are not sufficient to prevent the survival and growth of *L. monocytogenes*. If not killed by extreme stress, *L. monocytogenes* may either adjust to the stress conditions or, by means of flagellation, escape the harmful exposure by moving towards a more benign environment (Galsworthy *et al.*, 1990, Wright *et al.*, 2014).

2.5.1 General stress responses and stress tolerance mechanisms

Low temperature

L. monocytogenes is a psychrotrophic organism able to resist a cold milieu. Environmental bacteria may encounter low temperatures in their natural habitat, while foodborne pathogens also experience cold stress during the storage and refrigeration of vehicle foods. Temperature downshift has multiple direct and indirect impacts on bacteria, such as a loss of membrane fluidity and molecule solubility, slow nutrient transport and diffusion, increased RNA and DNA secondary structure stability, a decelerated lag phase and growth rate, and a restricted function of enzymes and other proteins, as well as direct protein damage (Beales, 2004, Tasara and Stephan, 2006, Chan and Wiedmann, 2009, Soni *et al.*, 2011).

L. monocytogenes is particularly capable of modulating its membrane lipids, which consist of an unusually high proportion of odd-numbered, iso and anteiso, branched-chain fatty acids (FAs) (Raines *et al.*, 1968, Annous *et al.*, 1997). In response to a decrease in temperature, multiple alterations in membrane lipid structure take place to allow the cell to maintain membrane fluidity and, consequently, to uphold vital functions in regard to enzyme activity and cross-membrane transportation (Beales, 2004, Tasara and Stephan, 2006, Chan and Wiedmann, 2009, Soni *et al.*, 2011). One of the key changes is the increased relative proportion of $C_{15:0}$ FAs and, in turn, a decrease in $C_{17:0}$ (Püttmann *et al.*, 1993, Russell *et al.*, 1995, Annous *et al.*, 1997, Mastronicolis *et al.*, 1998). As $C_{15:0}$ outnumbers $C_{17:0}$, the proportion of shorter FAs rises, which decreases the interaction between carbon chains, lowers their melting temperature, and thereby retains membrane fluidity (Russell *et al.*, 1995, Beales, 2004). In addition, unsaturation due to a slight increase in $C_{18:1}$ supports the fluid structure (Russell *et al.*, 1995). Annous *et al.* (1997) stated the shift from iso to anteiso branching, particularly to anteiso-$C_{15:0,}$ to be of importance in the growth of *L. monocytogenes* at cold temperatures.

In addition to physical changes within the cell membrane, *L. monocytogenes* is able to actively transport and accumulate compatible solutes from its environment into the cell and harness them as cryoprotectants without inhibiting other vital cellular functions (Wilkins *et al.*, 1972, Tasara and Stephan, 2006, Chan and Wiedmann, 2009). Upon cold stress, the expression of the transporter-encoding genes and thereby the uptake of carnitine and glycine betaine is induced (Angelidis and Smith, 2003, Wemekamp-Kamphuis *et al.*, 2004a, Miladi *et al.*, 2017). Carnitine is mainly transported into the cell by the OpuC transporter and glycine betaine by Gbu, and to a lesser extent by BetL (Angelidis and Smith, 2003). These compatible solutes markedly stimulate the growth of cold-stressed *L. monocytogenes* cells (Beumer *et al.*, 1994, Ko *et al.*, 1994, Wemekamp-Kamphuis *et al.*, 2004a).

One of the key responses of psychrotrophic bacteria to low temperatures is the recruitment of cold shock proteins (Csps), synthesized upon an abrupt

decrease in temperature, and cold acclimation proteins (Caps), produced in balanced growth at cold temperatures (Hébraud and Potier, 1999). Bayles *et al.* (1996) recognised twelve Csps whose synthesis was induced in *L. monocytogenes* 10403S following a temperature downshift from 37 °C to 5 °C. Four of these were also identified as Caps (Bayles *et al.*, 1996). At 4 °C, the production of 32 proteins designated as Csps was induced ≥3-fold in *L. monocytogenes* EGD (Phan-Thanh and Gormon, 1995). Cold shock protein CspA, CspB and CspD-encoding genes were upregulated at 4 °C and, of these proteins, CspA proved to be required for the growth of *L. monocytogenes* at both 4 °C and 10 °C (Schmid *et al.*, 2009). While a core set of 22 Cap-encoding genes was induced from the early lag to the late stationary phase, the cold-stress regulon of *L. monocytogenes* has been shown to be highly variable across different growth phases, implicating a growth phase-dependent response to low temperatures (Hingston *et al.*, 2017a).

Besides Csps and Caps, induction of the expression and production of multiple genes and proteins, respectively, has been reported in *L. monocytogenes* upon temperature downshift, indicating diverse mechanisms contributing to cold tolerance (Liu *et al.*, 2002, Chan *et al.*, 2007b, Cacace *et al.*, 2010). Liu *et al.* (2002) detected 11 genes whose expression was induced at 10 °C. Among these, ferritin-like cold-adaptive response protein, encoded by *flp*, has been named both as Csp and Cap (Bayles *et al.*, 1996, Hébraud and Guzzo, 2000). Other induced genes encode the general stress response proteins ClpB and ClpP and proteins involved in regulatory adaptive responses, alterations in the cell surface, and amino acid and degradative metabolism (Liu *et al.*, 2002). Chan *et al.* (2007b) reported the upregulation of genes that encode lipid and carbohydrate metabolism, motility, transcription and translation-related proteins at 4 °C, further illustrating the versatility of cold stress responses. Altogether 105 and 170 genes were ≥2-fold transcribed in log- and stationary-phase cells, respectively (Chan *et al.*, 2007b). Furthermore, several TCSs (reviewed in chapter 2.5.4) play an important role in sensing low temperature and mediating the cold stress response (Chan *et al.*, 2007b, Chan *et al.*, 2008). DEAD-box RNA helicases have a pivotal function in RNA metabolism and translation initiation (Chan and Wiedmann, 2009, Linder and Jankowsky, 2011). The DEAD-box proteins Lmo0866, Lmo1450 and Lmo1722 play a role in the growth and motility of *L. monocytogenes* at low temperature (Chan *et al.*, 2007b, Markkula *et al.*, 2012b). Regarding motility and flagellation, flagellin-encoding *flaA* presented elevated transcript levels at 10 °C (Liu *et al.*, 2002). The *flhA* and *motA* genes encoding the temperature-sensitive flagella synthesis proteins FlhA and MotA, respectively, contribute to the growth and motility of *L. monocytogenes* at 3 °C (Mattila *et al.*, 2011).

Alternative stress sigma factor σ^B, encoded by *sigB*, coordinates many stress responses in *L. monocytogenes* (Becker *et al.*, 1998, Becker *et al.*, 2000, Kazmierczak *et al.*, 2003, Raengpradub *et al.*, 2008) and directly and

positively regulates over 140 genes (Raengpradub *et al.*, 2008). The activity of σ^B is induced in stationary-phase cells upon temperature downshift (Becker *et al.*, 2000). Deletion of *sigB* renders the cell unable to accumulate betaine and carnitine (Becker *et al.*, 2000). Also, carnitine transporter-encoding *opuCA* is at least partly σ^B-dependent (Chan *et al.*, 2007a), further suggesting a role for σ^B in harnessing compatible solutes. On the other hand, the transcription of *opuCA* as well as of cold stress genes *fri* and *oppA* is independent of σ^B during cold stress at 4 °C (Chan *et al.*, 2007a). Although supporting the cold stress response, it was further demonstrated that σ^B is not unequivocally essential for the growth of *L. monocytogenes* at 4 °C (Chan *et al.*, 2007a).

High temperature

Although a psychrotrophic organism, *L. monocytogenes* tolerates fairly high temperatures, particularly as compared to other nonspore-forming foodborne pathogens, and may survive mild heat treatments (Doyle *et al.*, 1987, Doyle *et al.*, 2001, van der Veen *et al.*, 2007). Foodborne pathogens frequently encounter high temperatures during food processing. When subjected to heat reaching lethal temperatures, the DNA structure, ribosomes and ribosomal RNA degrade, the cytoplasmic membrane is damaged leading to the leakage of cellular components, and protein denaturation is initiated, resulting in metabolic malfunctions and reduced enzyme activity (Russell, 2003, Soni *et al.*, 2011). Thus, to maintain cellular mechanisms and to survive high temperatures, cells need to preserve nucleic acid functions, repair membranes and produce new proteins while removing damaged ones (van der Veen *et al.*, 2009, Soni *et al.*, 2011).

To overcome heat shock, *L. monocytogenes* harnesses two specific class I and III heat shock response mechanisms and a class II general stress response (van der Veen *et al.*, 2007). Class I contains an operon of classical chaperon-encoding *hrcA*, *grpE*, *dnaK*, *dnaJ*, *groEL* and *groES* genes (Hanawa *et al.*, 2000). The operon is negatively regulated by HrcA, which binds at a conserved inverted repeat element (Zuber and Schumann, 1994, Hanawa *et al.*, 2000, Hu *et al.*, 2007a). Class III heat shock proteins (Hsps) are comprised of Clp ATPases that function as ATP-dependent proteases, breaking down damaged and misfolded proteins, and as chaperons, aiding in protein folding (Wawrzynow *et al.*, 1996). Class III genes are negatively regulated by the transcriptional repressor CtsR (Derré *et al.*, 1999, Nair *et al.*, 2000, Hu *et al.*, 2007b). Besides stress tolerance, the chromosomally encoded ClpC, ClpP and ClpE class III Hsps are involved in the virulence of *L. monocytogenes* (Nair *et al.*, 2000, Rouquette *et al.*, 1996). Class II, positively regulated by σ^B, is comprised of diverse genes including listeriolysin positive regulatory protein-encoding *prfA* and general stress protein-encoding *ctc* (Benson and Haldenwang, 1993, van der Veen *et al.*, 2007). Despite the positive regulation of class II by σ^B, heat stress resistance

in *L. monocytogenes* seems to be at least partly independent of σ^B alone (Ferreira *et al.*, 2001).

Marked overlap has been detected within HrcA, CtsR and σ^B regulons, demonstrating a large regulatory network contributing to heat stress tolerance in *L. monocytogenes* (Hu *et al.*, 2007a, Hu *et al.*, 2007b, Raengpradub *et al.*, 2008, Chaturongakul *et al.*, 2011). Five genes were identified to be coregulated by all three regulators (Hu *et al.*, 2007a), and σ^B was detected to coregulate 25 and 21 genes with CtsR and HrcA, respectively (Chaturongakul *et al.*, 2011). In addition, σ^B contributes to the positive regulation of HrcA-encoding *hrcA* (Hu *et al.*, 2007a).

pH stress

L. monocytogenes encounters low pH throughout the food chain and within hosts: in silage, in fermented and other low-pH foods, during gastric passage in a host and inside macrophages upon phagocytosis (Beauregard *et al.*, 1997, Ryser *et al.*, 1997, Cotter *et al.*, 2001a, Cotter *et al.*, 2001b, Cotter and Hill, 2003). Alkaline conditions are commonly encountered in contact with detergents, but also upon pancreatic secretion in the host small intestine (Giotis *et al.*, 2007a, Soni *et al.*, 2011).

Acid stress reduces the growth rate and yield (Cheroutre-Vialette *et al.*, 1998, Bowman *et al.*, 2012). Weak organic acids are more harmful to bacterial cells compared to strong acids. They are membrane-permeable in their undissociated form and, once intracellular, weak acids dissociate, release a proton and directly acidify the intracellular space. Strong acids nevertheless lower the external pH and damage enzymes and, as proton permeability is induced due to a high gradient, strong acids may also ultimately acidify the cytoplasm, albeit less drastically than weak acids (Phan-Thanh *et al.*, 2000, Cotter and Hill, 2003, Beales, 2004). Alkaline conditions, in turn, disturb cell division and growth, elongate listerial cells and damage cell membranes, leading to the leakage of intracellular components (Zilberstein *et al.*, 1984, Sampathkumar *et al.*, 2003, Giotis *et al.*, 2007a).

Upon pH stress, bacterial cells need to maintain pH homeostasis: proton extrusion under acidic and inward transport of protons under alkaline conditions (Zilberstein *et al.*, 1984, Shabala *et al.*, 2002, Krulwich *et al.*, 2011). Transient failure of pH control may precede the reestablishment of the intracellular pH (Zilberstein *et al.*, 1984). Besides alterations in transport and metabolic functions, surmounting both high and low pH involves structural changes in cell composition (Krulwich *et al.*, 2011). In *L. monocytogenes*, the proportion of anteiso FAs increases at an alkaline pH, while the opposite takes place under acidic conditions (Giotis *et al.*, 2007b). The proportion of iso and anteiso forms is putatively of more importance in pH adaptation than changes in the saturation of FAs (Giotis *et al.*, 2007b).

Membrane transport functions are pivotal in maintaining intracellular pH homeostasis in *L. monocytogenes*, particularly under acid stress (Cotter and

Hill, 2003). As a facultative anaerobic bacterium, *L. monocytogenes* may utilise both the active transport of protons, through a H^+-linked electron transport system in the aerobic respiratory chain, and transport via H^+-ATPase molecules, including F_0F_1-ATPase, which use energy anaerobically derived from ATP hydrolysis (Cotter *et al.*, 2000, Shabala *et al.*, 2002). To maintain the intracellular pH under acidic conditions, *L. monocytogenes* employs the glutamate decarboxylase system (GAD), encoded by *gadA*, *gadB* and *gadC* (Cotter *et al.*, 2001a, Cotter *et al.*, 2001b). Another GAD and an additional antiporter is encoded by *gadD* and *gadE*, respectively (Wemekamp-Kamphuis *et al.*, 2004b). GAD consumes intracytoplasmic protons by producing γ-aminobutyrate (GABA) from extracellular glutamate, hence elevating the cytoplasmic pH (Small and Waterman, 1998). GABA is then released, and the substitution of glutamate with GABA outside the cell will also slightly alkalinise the extracellular environment (Small and Waterman, 1998). In addition to GAD, *L. monocytogenes* harbours a functional arginine deiminase system (ADI), encoded by *arcA*, *arcB*, *arcC* and *arcD*, also playing a role in the acid tolerance of *L. monocytogenes* (Ryan *et al.*, 2009). The σ^B mutant displays impaired resistance of *L. monocytogenes* to acid stress, indicating the importance of σ^B in surmounting acidic conditions (Wiedmann *et al.*, 1998, Ferreira *et al.*, 2001, Wemekamp-Kamphuis *et al.*, 2004b). Furthermore, the expression of the *gadCB* operon and ADI system is regulated by σ^B (Kazmierczak *et al.*, 2003, Wemekamp-Kamphuis *et al.*, 2004b, Ryan *et al.*, 2009). During acid adaptation at pH 5.5 and acid stress at pH 3.5, the synthesis of 37 and 47 proteins, respectively, was induced ≥2-fold as compared to a neutral pH of 7.2 (Phan-Thanh and Mahouin, 1999). The number of proteins may reflect the increasing effort to maintain intracytoplasmic pH homeostasis under harshening acid stress (Phan-Thanh and Mahouin, 1999).

While the exact mechanisms allowing *L. monocytogenes* to tolerate high pH levels remain fairly obscure, upon alkali stress, *L. monocytogenes* appears to strive at diminishing excess alkalisation and energy outlay whilst mobilising carbon sources (Giotis *et al.*, 2008b). Transcriptional profiles were found to differ between abrupt alkali shock and alkali adaptation (Giotis *et al.*, 2010). Giotis *et al.* (2008b) found the synthesis of 45 proteins to be induced ≥1.5-fold upon alkali adaptation, including DnaK and GroEL (Giotis *et al.*, 2008b). Alkali stress seems to mimic phosphate starvation in *L. monocytogenes*, and phosphate uptake and utilisation appears to be revised upon alkali exposure (Giotis *et al.*, 2008b).

Osmotic stress

Foodborne pathogens frequently encounter osmotic stress due to high salt concentrations, as sodium chloride is commonly used as a food preservative and flavour additive. Desiccation and accumulating osmotically active substances leads to a decrease in water activity (a_w), the water quantity available to bacterial cells (Gutierrez *et al.*, 1995, Beales, 2004).

Consequently, the cytoplasmic volume decreases and cells begin to lose their intracellular turgor pressure, cellular functions deteriorate, and bacterial growth decelerates (Csonka, 1989, Cheroutre-Vialette *et al.*, 1998, Beales, 2004). Bacteria resist these changes through osmoadaptation (Galinski, 1995, Gutierrez *et al.*, 1995).

Under osmotic stress, listerial cells try to maintain the proper lipid bilayer of their cell membrane by increasing the proportion of anionic lipids, particularly diphosphatidylglycerol (Russell *et al.*, 1995). Furthermore, cells restore their internal turgor pressure by accumulating from their environment the same compatible solutes, glycine betaine and carnitine, as under cold conditions (Csonka, 1989, Patchett *et al.*, 1992, Gutierrez *et al.*, 1995, Bayles and Wilkinson, 2000). The compatible solutes stabilise intracellular enzymes (Russell *et al.*, 1995) and potentially hydrate the protein surfaces, stabilising proteins and preventing their misfolding (Kempf and Bremer, 1998). They also induce the growth of *L. monocytogenes* upon osmotic stress and a_w decrease (Beumer *et al.*, 1994, Bayles and Wilkinson, 2000). In 4% NaCl, a 200-fold increase in the transportation rate of glycine betaine has been reported (Ko *et al.*, 1994), and associated loci are upregulated even under milder osmotic stress (1.2% NaCl) (Bae *et al.*, 2012). Proline also serves as an osmolyte in *L. monocytogenes* (Sleator *et al.*, 2001). Besides free amino acids, peptides aid in maintaining the cell turgor and thereby contribute to osmotolerance (Amezaga *et al.*, 1995). While proline only mediates osmotolerance at high concentrations (10 mM) (Beumer *et al.*, 1994), as a peptide, it confers osmoprotection even as a mild (1 mM) solution (Amezaga *et al.*, 1995).

Upon osmotic stress, Duché *et al.* (2002) detected six rapidly induced (<30 min) salt shock proteins (Ssps) and eleven salt acclimation proteins (Saps) that are more slowly induced (60–90 min) but maintain the induced level for a prolonged period (Duché *et al.*, 2002a). The Ssps included heat shock protein DnaK, general stress response protein Ctc, and proteins involved in general metabolism (Duché *et al.*, 2002a, Duché *et al.*, 2002b). Glycine betaine transporter Gbu was induced later during the adaptational phase (Duché *et al.*, 2002a). Ctc is putatively involved in osmotolerance, regardless of osmoprotectants, as glycine betaine is able to ameliorate the detrimental osmotic effects similarly in both the wild type and *ctc* mutant (Gardan *et al.*, 2003).

σ^B also regulates multiple stress response genes in the osmotolerance of *L. monocytogenes* (Becker *et al.*, 1998, Kazmierczak *et al.*, 2003, Abram *et al.*, 2008, Ribeiro *et al.*, 2014). The osmotic induction of carnitine transporter-encoding *opuC* is σ^B-dependent (Fraser *et al.*, 2003), and the absence of σ^B also diminishes the uptake of betaine, suggesting σ^B to play a role in osmolyte transportation upon osmotic stress in *L. monocytogenes* (Becker *et al.*, 1998). In response to salt stress, σ^B represses the transcription of flagellar protein-encoding genes, indicating an indirect impact on motility and chemotaxis (Raengpradub *et al.*, 2008). Although chemotaxis allows

bacterial movement to more favourable surroundings, flagellar synthesis is an energy-consuming process, and downregulation of flagellar genes may be a means for bacterial cells to allot energy to more crucial mechanisms needed to surmount osmotic stress (Hingston *et al.*, 2015). Additionally, genes encoding RNA helicases (Markkula *et al.*, 2012a) and TCSs (Kallipolitis and Ingmer, 2001) are involved in the growth of *L. monocytogenes* under osmotic stress.

Ethanol stress

Foodborne bacteria frequently encounter high ethanol concentrations during sanitation and preservation processes and in some food products. While Gram-negative bacteria seem to be fairly susceptible to ethanol, Gram-positives and particularly lactic acid bacteria survive markedly high ethanol concentrations (Ingram, 1976, Gold *et al.*, 1992, Silveira *et al.*, 2004, van Bokhorst-van de Veen *et al.*, 2011). *Lactobacilli* can grow in 10% ethanol and intermediate growth of some strains has even been observed in 16% (Gold *et al.*, 1992). In *L. monocytogenes*, a 5% ethanol concentration seems to inhibit growth (Oh and Marshall, 1993). Ethanol may also augment the lethal effects of concomitantly administered acids and salt on *L. monocytogenes*, possibly associated with cell membrane damage (Barker and Park, 2001).

Ethanol increases membranous permeability in bacterial cells, both by altering the aqueous environment and by impairing the hydrophobic membrane core barrier. Being freely permeable through the cell membrane, ethanol affects not only enzymes on cell surfaces and within membranes but also in the cytoplasm (Ingram, 1990). Ethanol hampers protein folding and macromolecular interactions, consequently inhibiting membrane protein synthesis and decelerating cell growth (Ingram, 1990, Seydlová *et al.*, 2012). Growth inhibition also emanates from the fluidising effect of ethanol on lipid bilayers, which may further lead to a compensatory increase in membrane rigidity (Dombek and Ingram, 1984, Huffer *et al.*, 2011, Seydlová *et al.*, 2012). Commonly, an increase in saturated and unbranched FAs within the cell membrane occurs upon ethanol stress (Huffer *et al.*, 2011). In *Bacillus subtilis*, ethanol exposure and adaptation leads to the recruitment of molecular chaperones in order to restore phospholipid synthesis and membrane fluidity (Seydlová *et al.*, 2012).

The impacts of ethanol on microbial cells are thought to derive from mechanisms affecting hydrophobic associations and from the colligative impact rather than via specific receptors (Ingram, 1976, Ingram, 1990). However, it has been shown that ethanol induces the termination of Rho-dependent transcription in *E. coli* and inhibits the mRNA synthesis and translation of nonstart AUG codons, indicating a significant impact on the transcriptional and translational functions upon ethanol stress (Haft *et al.*, 2014). Haft *et al.* (2014) further suggested that alterations in *E. coli* membranes upon ethanol exposure might partly be due to ethanol directly affecting ribosomes and RNA polymerases.

In addition to genes encoding proteins directly applied to coping with high ethanol concentrations, such as alcohol and aldehyde dehydrogenases YqhD and AldB, respectively (Soufi *et al.*, 2015), many general stress response genes are induced upon ethanol stress. In *B. subtilis*, a promoter of extracytoplasmic function sigma factor σ^M was induced under ethanol stress (Thackray and Moir, 2003). In *L. monocytogenes*, RNA helicase Lmo0866 and RNA-binding protein Hfq have been shown to play roles in ethanol tolerance (Christiansen *et al.*, 2004, Markkula *et al.*, 2012a). The expression of *hfq* was, furthermore, found to be σ^B-dependent upon ethanol stress (Christiansen *et al.*, 2004).

Oxidative stress

L. monocytogenes cells experience oxidative stress both in the external environment and during intracellular host infection (Beckerman *et al.*, 1993, Gorski *et al.*, 2008, Soni *et al.*, 2011). Reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2), are metabolic by-products but also derive from external chemical processes or are released by competing microorganisms as a defence mechanism (Mishra and Imlay, 2012, Imlay, 2015a, Imlay, 2015b). As very reactive agents, ROS damage enzymes, other proteins and nucleic acids, and are harmful to cell lipid membranes. Consequently, ROS sensing and detoxification and the induction of cellular repair mechanisms are pivotal in surmounting oxidative stress (Cabiscol *et al.*, 2000, Imlay, 2002, Lee and Helmann, 2006, Mishra and Imlay, 2012, Imlay, 2015a).

The predominant regulator of the oxidative stress response in Gram-positive bacteria is PerR, a repressor of the oxidative response gene regulon (Chen *et al.*, 1995, Imlay, 2015b). In *B. subtilis*, PerR regulates the expression of genes for catalase KatA, alkyl hydroperoxide reductase AhpCF, the heme synthesis operon HemAXCDBL, the zinc uptake system ZosA, the ferric uptake repressor Fur, and protective DNA-binding Dps encoded by *mrgA* (Chen *et al.*, 1995, Helmann *et al.*, 2003). PerR and Dps-like Fri also facilitate the oxidative stress response in *L. monocytogenes* (Dussurget *et al.*, 2005, Olsen *et al.*, 2005, Rea *et al.*, 2005). The PerR regulon in *L. monocytogenes* includes *fri*, *fur*, *hemA*, *kat*, *trxB*, *lmo0641* and *lmo1604* (Rea *et al.*, 2005). Of these, *lmo0641* is the closest homolog of *zosA* in *B. subtilis* PerR-regulated genes, while *lmo1604* displays 32% identity with *ahpC*, and *fri* 35% identity with *mrgA* (Rea *et al.*, 2005), indicating a similar regulon to that described in *B. subtilis*. PerR senses peroxides by metal-catalysed oxidation (Lee and Helmann, 2006). It responds to low levels of H_2O_2 when the metal-binding site of PerR is occupied by Fe(II) while being less sensitive to H_2O_2 when occupied by Mn(II) (Herbig and Helmann, 2001). Although PerR is a direct sensor of H_2O_2, *perR* undergoes little induction by H_2O_2 (Fuangthong *et al.*, 2002). However, apart from *perR* and *fur*, other PerR regulon genes are induced by H_2O_2 (Fuangthong *et al.*, 2002).

Mutational analyses have revealed roles for superoxide dismutase, σ^B, and RNA helicase Lmo1450 in the viability of *L. monocytogenes* upon oxidative

stress (Ferreira *et al.*, 2001, Archambaud *et al.*, 2006, Markkula *et al.*, 2012a). Under cold conditions, elevated levels of transcripts and the induction of proteins involved in protection against oxidative stress, such as thioredoxin reductase-encoding *trxB*, ferritin-like *flp*, superoxide dismutase and catalase, have been detected, suggesting *L. monocytogenes* to undergo concomitant oxidative stress at low temperatures (Liu *et al.*, 2002, Cacace *et al.*, 2010).

Benzalkonium chloride

Benzalkonium chloride (BC) belongs to the group of quaternary ammonium compounds (QAC), cationic surfactants widely used as detergents for disinfection on both organic and abiotic surfaces (McDonnell and Russell, 1999). The antimicrobial attributes of QACs are proposed to be based on the generalised disruption of cytoplasmic cell membranes, followed by a loss of structural integrity, degradation of proteins and nucleic acids and leakage of intracellular matrix, as well as cell wall lysis (Denyer, 1995, McDonnell and Russell, 1999). BC has also been shown to inhibit biofilm formation (Houari and Di Martino, 2007).

Repeated exposure to sublethal concentrations of detergents and disinfectants may result in emerging resistance of bacterial strains to these compounds (Lundén *et al.*, 2003a, Houari and Di Martino, 2007). Concerningly, BC-resistant (BC^r) strains of *L. monocytogenes* have been isolated from both food-processing environments (Mullapudi *et al.*, 2008) and foodstuffs (Soumet *et al.*, 2005), as well as clinical cases (Elhanafi *et al.*, 2010). Approximately 10% to 46% of *L. monocytogenes* isolates from foodstuffs and processing environments have been reported as BC-resistant (Aase *et al.*, 2000, Mullapudi *et al.*, 2008). However, it should be noted that the definition of BC resistance varies between studies. For instance, Aase *et al.* (2000) and Soumet *et al.* (2005) considered the strains with a minimal inhibitory concentration (MIC) of BC of more than 4 µg/ml and 7.5 µg/ml, respectively, to be BC^r, while others have estimated a BC resistance threshold of ≥10 µg/ml (Mullapudi *et al.*, 2008, Dutta *et al.*, 2013, Jiang *et al.*, 2016).

Genes known to confer BC resistance in *L. monocytogenes* include the efflux pump systems-encoding *bcrABC* (Elhanafi *et al.*, 2010), *emrE* (Gilmour *et al.*, 2010, Kovacevic *et al.*, 2016), and *qacH* on the transposon Tn*6188* (Müller *et al.*, 2013). While *L. monocytogenes* strains harbour BC resistance-mediating genes, such as *bcrABC*, on the chromosome (Dutta *et al.*, 2013), plasmid-borne *bcrABC* has also been detected (Elhanafi *et al.*, 2010, Dutta *et al.*, 2013, Jiang *et al.*, 2016), indicating that BC resistance may be efficiently acquired and spread via horizontal gene transfer. The efflux pump MdrL-encoding *mdrL*, found to be both chromosomal and plasmid-borne (Romanova *et al.*, 2002), has been proposed at least with a partial role in the adaption of *L. monocytogenes* to BC (Mereghetti *et al.*, 2000, Romanova *et al.*, 2006). Previously, the chromosomal MdrL, negatively regulated by LadR, was shown to play a role in the survival of

L. monocytogenes at both lethal and sublethal BC concentrations (Jiang *et al.*, 2019).

Cross-adaptation

Exposure to certain stress conditions may enhance the tolerance of *L. monocytogenes* to subsequent exposure to the same or different stressors (Lou and Yousef, 1997, Lundén *et al.*, 2003a, Skandamis *et al.*, 2008, Soni *et al.*, 2011). Cross-protection has potentially grave implications for food safety, as multiple stress conditions are often concurrently used in food processing to prevent bacterial growth. Under many stresses, preadaptation to sublethal stress increases resistance to more severe conditions of the same media. Acid adaptation has been widely shown to augment resistance in *L. monocytogenes* to subsequent exposure to a lethal acidic pH (Kroll and Patchett, 1992, Hill *et al.*, 1995, O'Driscoll *et al.*, 1996, Lou and Yousef, 1997, Phan-Thanh *et al.*, 2000). Alkali-adapted cells, in turn, are more resistant to harsher alkaline stress (Giotis *et al.*, 2008a). Adaptational augmentation in resistance has also been demonstrated under ethanol, oxidative (Lou and Yousef, 1997, Ferreira *et al.*, 2001) and heat stresses (Shen *et al.*, 2014).

Reciprocal cross-protection under different stress conditions has been described in *L. monocytogenes*, as osmoadaptation has been found to increase acid resistance (Faleiro *et al.*, 2003), while acid tolerance, in turn, provides cross-protection against osmotic shock (Phan-Thanh *et al.*, 2000, Faleiro *et al.*, 2003). Similarly, heat-adapted cells are more resistant to acid shock (Phan-Thanh *et al.*, 2000), while acid adaptation leads to increased heat resistance (Farber and Pagotto, 1992, Phan-Thanh *et al.*, 2000).

Lou and Yousef (1997) revealed a complicated net of adaptational responses and cross-protection in *L. monocytogenes*. Resistance to lethal oxidative stress was enhanced by sublethal exposure to ethanol, acid, osmotic and heat stress. Additionally, survival under ethanol stress was increased by preceding acid and heat exposure, acid resistance improved following ethanol adaptation, and resistance to osmotic stress increased following preadaptation to ethanol and heat (Lou and Yousef, 1997). In addition to low temperature treatment providing cross-protection against mild osmotic stress, a prolonged duration of cold preadaptation was found to progressively enhance the survival of *L. monocytogenes* under subsequent osmotic stress (Pittman *et al.*, 2014). Furthermore, acid- as well as alkali-adapted cells display enhanced resistance to heat, osmotic and alcohol stresses (Phan-Thanh *et al.*, 2000, Taormina and Beuchat, 2001, Giotis *et al.*, 2008a).

Extensive cross-protection under various stresses may be explained by the recruitment of many of the same response mechanisms and induction of the same proteins under different conditions. *L. monocytogenes* employs versatile genetic mechanisms and molecular determinants to comprehensively contend with stress conditions and to persist in and adapt to niche-specific environments. Particular stress survival islets (SSI) have been recognised to play roles under multiple suboptimal conditions: a self-

regulating five-gene (*lmo0444–lmo0448*) stress survival islet, SSI-1, contributes to the growth of *L. monocytogenes* under acid and osmotic stresses (Ryan *et al.*, 2010), while SSI-2, mainly harboured by sequence type 121 (ST121) *L. monocytogenes* strains, has a role under alkaline and oxidative stresses (Harter *et al.*, 2017). Survival under harsh stresses is energy-consuming, and proteins involved in the ATP synthase chain and oxidative phosphorylation are thus commonly induced upon stress adaptation (Pittman *et al.*, 2014). Bergholz *et al.* (2012) found 888 genes with temperature-dependent alterations in transcript levels during osmotic adaptation. Based on proteomic analysis, Pittman *et al.* (2014) identified 299 differentially expressed proteins associated with the osmotic response of cold-adapted cells, pinpointing proteins putatively involved in cross-protection. Amongst others, these included cell envelope proteins and proteins involved in metabolic pathways (Pittman *et al.*, 2014). Regarding cellular processes, compatible solutes are needed for viability under both cold and osmotic stress (Bayles and Wilkinson, 2000). The alternative stress sigma factor σ^B and σ^B-regulated genes also play roles under multiple stresses (Bergholz *et al.*, 2012). The expression of SSI-1 genes was shown to be regulated by σ^B (Ryan *et al.*, 2010). Besides cold tolerance, cold shock proteins CspA, CspB and CspD contribute to some extent to osmotic and oxidative stress tolerance (Schmid *et al.*, 2009, Loepfe *et al.*, 2010). Many heat stress proteins mediate tolerance to other stresses, such as HtrA to osmotic, acid and oxidative stress (Wonderling *et al.*, 2004, Stack *et al.*, 2005, Wilson *et al.*, 2006) and DnaK and GroEL to alkaline stress (Giotis *et al.*, 2008b). Phan-Thanh and Gormon (1995) also found six proteins to be induced by both heat and cold shock.

2.5.2 Strain diversity in stress responses

L. monocytogenes strains do not form a homogeneous population but vary in their phenotypic characteristics (Abee *et al.*, 2016, Grad and Fortune, 2016). Certain lineages and serotypes display particular resistance or sensitivity to stresses frequently encountered along the food chain. Salt stress tolerance phenotypes vary according to lineage, as lineage I strains and III grow faster under osmotic stress than those of lineage II (Bergholz *et al.*, 2010, Ribeiro and Destro, 2014). Higher transcript levels of σ^B-dependent *gadD* and CtsR-dependent *clpB* were found in lineage I strains as compared to lineage II strains post-saline shock (Ringus *et al.*, 2012). Serotype 4b isolates have proven to be more heat-sensitive than other serotypes (Lianou *et al.*, 2006), while presenting a higher tolerance to low pH and high salt concentrations (van der Veen *et al.*, 2008). However, under heat and acid stresses, death rates have also been shown to vary markedly between strains (Mackey *et al.*, 1990, Lianou *et al.*, 2006). In the next-generation sequencing (NGS) era, serotypes 1/2a and 1/2b have presented on average more tolerant phenotypes under cold conditions than 4b and 1/2c (Hingston *et al.*, 2017b). Strain-

dependent behaviour is also seen in biofilm formation and in the growth of *L. monocytogenes* at low temperatures, low a_w and in vacuum packaging (Barbosa *et al.*, 1994, Barbosa *et al.*, 1995, Begot *et al.*, 1997, Nilsson *et al.*, 2011, Cordero *et al.*, 2016). Differences in stress tolerance have also been associated with the isolation sources of *L. monocytogenes* isolates. Clinical strains seem to display greater resistance to acid and osmotic stresses than environmental and food-related strains (Dykes and Moorhead, 2000, Vialette *et al.*, 2003).

Besides strain variability, population heterogeneity, i.e. variability between single cells of a strain or in a population, describes the diverse stress tolerance of pathogenic bacteria and may have further implications for food safety (Lianou and Koutsoumanis, 2013, Abee *et al.*, 2016, den Besten *et al.*, 2017). Heterogeneity may indeed benefit cell survival and thus be of advantage to *L. monocytogenes* under stressful conditions (Metselaar *et al.*, 2013). Furthermore, strain variability as well as population heterogeneity seem to increase along with harshening stress conditions (Francois *et al.*, 2006, Lianou and Koutsoumanis, 2013). Thus, in addition to unravelling specific traits rendering single strains tolerant, it is increasingly pivotal to comprehensively address strain variability in food hygiene research.

2.5.3 Plasmid-mediated stress tolerance in *L. monocytogenes*

Plasmids, like other MGEs, are profoundly self-seeking entities and are thought to essentially operate irrespective of the needs of their hosts (Rankin *et al.*, 2011). Carrying a plasmid may be costly for the host cell due to the synthesis of proteins needed in plasmid replication and transmission. However, plasmids frequently harbour genes that may engender fitness in the host and may even benefit the neighbouring cells by degrading harmful compounds (Kado, 1998, Löfmark *et al.*, 2008, Slater *et al.*, 2008, Rankin *et al.*, 2011). Plasmids may also provide their hosts with adaptive traits conferring enhanced antibiotic resistance or virulence (Poirel *et al.*, 2007, Herrero *et al.*, 2008, Périchon *et al.*, 2008, Carattoli *et al.*, 2010, Venturini *et al.*, 2010). Intriguingly, conjugative natural antibiotic resistance plasmids have also been isolated in commensal organisms, suggesting a resistance gene reservoir that may not be affected by a decrease in antibiotic usage and the following alleviation of selective pressure (Yates *et al.*, 2006). In *L. monocytogenes*, identical plasmids have been isolated from different geographic locations over significantly long time periods (Flamm *et al.*, 1984, Hingston *et al.*, 2017b), suggesting that these plasmids benefit their parental strains by improved resistance and survival: A conserved plasmid was found in four different strains isolated over a period of 12 years (Fox *et al.*, 2016).

Plasmids have been shown to confer tolerance of *L. monocytogenes* to stressors such as the disinfectant benzalkonium chloride (Elhanafi *et al.*, 2010, Rakic-Martinez *et al.*, 2011, Katharios-Lanwermeyer *et al.*, 2012, Jiang *et al.*, 2016, Kremer *et al.*, 2017, Zhang *et al.*, 2018) and heavy metals

(Lebrun *et al.*, 1994, McLauchlin *et al.*, 1997, Rakic-Martinez *et al.*, 2011, Katharios-Lanwermeyer *et al.*, 2012). Hingston *et al.* (2017b) discovered the presence of plasmids to associate with enhanced acid tolerance and cold sensitivity of *L. monocytogenes*, and Zhang *et al.* (2018) reported pLMSZ08 plasmid curing resulting in decreased salt stress tolerance. However, knowledge of plasmids mediating environmental stress tolerance is rather limited and should be broadened, given the grave implications it has for food safety.

Plasmid-mediated antibiotic resistance, particularly to last-resort antibiotics, poses an emerging worldwide threat to both human and animal health (Liu *et al.*, 2016, Bachiri *et al.*, 2017). Concerningly, plasmids conferring resistance to antibiotics such as quinolones and the last-resort antibiotic colistin have been found in *E. coli* isolated from environmental samples (Jørgensen *et al.*, 2017, Ranjbar and Farahani, 2017). In *L. monocytogenes*, naturally occurring plasmids were already associated with enhanced resistance to antibiotics such as chloramphenicol, erythromycin and other macrolides, lincosamides, streptomycin and tetracycline in the early 1990s (Poyart-Salmeron *et al.*, 1990, Hadorn *et al.*, 1993). Thus, besides enhanced environmental fitness within bacteria, potential cotransfer of plasmid-borne stress resistance genes with genes conferring virulence and antibiotic resistance is of great concern (Herrero *et al.*, 2008, Bojer *et al.*, 2010, Gullberg *et al.*, 2014, Fox *et al.*, 2016). Plasmids may augment the ability of pathogenic hosts to occupy auxiliary niches, which creates further opportunities to infect new hosts. Conjugational transfer has been demonstrated not only between *Listeria* spp. strains (Katharios-Lanwermeyer *et al.*, 2012, Korsak *et al.*, 2019) but also to and from other species, such as *Lactococcus* spp. (Guglielmetti *et al.*, 2009), *Enterococcus* spp., *Staphylococcus* spp. (Poyart-Salmeron *et al.*, 1990) and *Streptococcus* spp. (Pérez-Díaz *et al.*, 1982, Flamm *et al.*, 1984, Poyart-Salmeron *et al.*, 1990), indicating naturally occurring inter-species horizontal gene transfer.

2.5.4 Two-component systems in the stress responses of *L. monocytogenes*

Signalling through TCSs is a fundamental means for bacteria to respond to and cope with versatile stresses. TCSs have been appointed a role among others in the cold tolerance and adaptation of *Clostridium botulinum* (Lindström *et al.*, 2012, Derman *et al.*, 2013, Dahlsten *et al.*, 2014, Mascher *et al.*, 2014) and *B. subtilis* (Aguilar *et al.*, 2001), as well as *B. subtilis* heat tolerance (Takada *et al.*, 2018), in the growth and motility of *Yersinia pseudotuberculosis* under cold conditions (Palonen *et al.*, 2011), and in the osmotolerance of *E. coli* (Nakashima *et al.*, 1992, Cai and Inouye, 2002). In addition to environmental stresses, TCSs are of importance in antibiotic resistance and the survival of pathogenic bacteria during host infection

(Beier and Gross, 2006, Fernández *et al.*, 2010, Altamirano-Silva *et al.*, 2018, Cao *et al.*, 2018). The exact physiological signal triggering the function of a particular TCS often remains obscure, but signal molecules such as nitrate, O_2, K^+ and Mg^{2+} have been recognised (Krell *et al.*, 2010, Zschiedrich *et al.*, 2016). Specifically, changes in membrane fluidity and unsaturated FAs were recognised as the signals through which the TCS DesKR responds to cold stress in *B. subtilis* (Aguilar *et al.*, 2001).

TCSs with phenotypically confirmed roles in augmenting the tolerance of *L. monocytogenes* to environmental stress conditions closely related to food processing and hygiene are presented in Table 2. Besides heat tolerance, the orphan RR DegU (Lmo2515) is a pleiotropic temperature-responsive regulator needed for flagellar synthesis and motility and is also involved in biofilm formation and virulence (Knudsen *et al.*, 2004, Williams *et al.*, 2005b, Gueriri *et al.*, 2008). Intriguingly, DegU controls flagellar genes in an unphosphorylated state within the receiver domain (Mauder *et al.*, 2008). CheYA (Lmo0691/Lmo0692) is needed for the characteristic swarming motility of *L. monocytogenes* (Flanary *et al.*, 1999, Dons *et al.*, 2004, Williams *et al.*, 2005a). The *agr* quorum-sensing system of *L. monocytogenes*, including the TCS AgrCA (Lmo0050/Lmo0051), affects adherence on abiotic surfaces and therefore the early stages of biofilm formation (Rieu *et al.*, 2007). LiaSR (Lmo1021/Lmo1022), LisRK (Lmo1377/Lmo1378) and CesRK (Lmo2422/Lmo2421) are involved in cell-envelope stress and, accordingly, in the resistance of *L. monocytogenes* to cell wall-acting antibiotics, such as cephalosporins (Cotter *et al.*, 2002, Kallipolitis *et al.*, 2003, Gottschalk *et al.*, 2008, Collins *et al.*, 2012, Nielsen *et al.*, 2012). For instance, LiaSR responds to cell-envelope stress by upregulating membrane-associated genes and remodelling the composition of the cytoplasmic membrane (Fritsch *et al.*, 2011). Furthermore, LisRK controls the transcription of virulence gene *htrA* (Sleator and Hill, 2005). VirR (Lmo1745) regulates the modification of cell surface components and enhances cell invasion and virulence in *L. monocytogenes* (Mandin *et al.*, 2005). ResD (Lmo1948) confers resistance to pediocin, affects carbon source utilisation and controls respiration in *L. monocytogenes* (Larsen *et al.*, 2006).

Table 2. Two-component systems with phenotypically confirmed roles in enhancing the tolerance of *L. monocytogenes* to high and low temperatures, acid and alkali pH, and osmotic, ethanol and oxidative stresses.

Two-component system (HK/ RR) [a]	Stress condition	Phenotypic evidence [b]	Reference
AgrA; Lmo0051 (RR)	Osmotic	Reduced growth of deletion mutant in 3% and 5% NaCl (L)	(Garmyn *et al*., 2012)
YycF; Lmo0287 (RR)	Cold	Upregulated transcription at 4 °C (L, S)	(Chan *et al*., 2007b)
Lmo1060 (RR)	Cold	Reduced growth of deletion mutant at 4 °C (L)	(Chan *et al*., 2008)
Lmo1172 (RR)	Cold	Reduced growth of deletion mutant at 4 °C (L)	(Chan *et al*., 2008)
LisK; Lmo1378 (HK)	Acid	Reduced growth of deletion mutant at pH 3.5 (L)	(Cotter *et al*., 1999)
	Osmotic	Reduced growth of deletion mutant in 8% NaCl (L)	(Sleator and Hill, 2005)
LisR; Lmo1377 (RR)	Acid	Reduced growth of insertion mutant at pH 5.25 (lag-phase inoculum)	(Kallipolitis and Ingmer, 2001)
	Cold	Reduced growth of deletion mutant at 4 °C (L)	(Chan *et al*., 2008)
	Heat	Reduced growth of insertion mutant at 43.5 °C (L, S)	(Kallipolitis and Ingmer, 2001)
	Oxidative	Reduced growth of insertion mutant in 0.025% H_2O_2 (L)	(Kallipolitis and Ingmer, 2001)
VirR; Lmo1745 (RR)	Ethanol	Reduced growth of deletion mutant in 5% ethanol (L)	(Williams *et al*., 2005a)
ResD; Lmo1948 (RR)	Ethanol	Reduced growth of deletion mutant in 5% ethanol (L)	(Williams *et al*., 2005a)
CesR; Lmo2422 (RR)	Heat	Reduced growth of insertion mutant at 43.5 °C (L, S)	(Kallipolitis and Ingmer, 2001)
	Osmotic	Reduced growth of insertion mutant in 9% NaCl (L, S)	(Kallipolitis and Ingmer, 2001)
PhoP; Lmo2501 (RR)	Ethanol	Reduced growth of deletion mutant in 5% ethanol (L)	(Williams *et al*., 2005a)
DegU; Lmo2515 (RR)	Heat	Reduced growth of deletion mutant at 44 °C (L)	(Gueriri *et al*., 2008)
	Ethanol	Reduced growth of deletion mutant in 5% ethanol (L)	(Williams *et al*., 2005a)
KdpE; Lmo2678 (RR)	Heat	Reduced growth of insertion mutant at 43.5 °C (L, S)	(Kallipolitis and Ingmer, 2001)
	Osmotic	Reduced growth of insertion mutant in 2% and 9% NaCl (L, S)	(Kallipolitis and Ingmer, 2001, Brøndsted *et al*., 2003)

[a] HK, histidine kinase; RR, response regulator

[b] L, logarithmic growth phase; S, stationary growth phase

3 AIMS OF THE STUDY

The aims of this study were to investigate the genetic mechanisms conferring stress tolerance in *L. monocytogenes* and to decipher strain diversity in *L. monocytogenes* stress resistance.

The specific aims of this study were:

1. To assess the role of two-component-system histidine kinases in the growth and stress tolerance of *L. monocytogenes* EGD-e at high and low temperatures, under acid, alkali and oxidative conditions, and in high salt and ethanol concentrations (I, II);

2. To identify accessory genetic mechanisms and plasmid-borne genes conferring heat resistance in *L. monocytogenes* (III);

3. To investigate strain variability and the occurrence of resistance-conferring genes with respect to benzalkonium chloride resistance within 392 *L. monocytogenes* isolates of Finnish and Swiss origin (IV).

4 MATERIALS AND METHODS

4.1 *L. monocytogenes* strains, isolates and plasmids (I–IV)

The sequenced *L. monocytogenes* EGD-e (Glaser *et al.*, 2001) was used for genetic modifications of HKs (I, II), *L. monocytogenes* isolates AL4E and AT3E (Lundén *et al.*, 2008) for plasmid-mediated heat resistance studies (III) and *L. monocytogenes* 10403S (Bishop and Hinrichs, 1987) for horizontal gene transfer experiments (III). The strains and plasmids used in studies I–III are presented in Table 3. A total of 392 *L. monocytogenes* isolates were selected for study IV from the culture collections of the Department of Food Hygiene and Environmental Health of the University of Helsinki, Finland (n = 197) and the Swiss National Reference Centre for Enteropathogenic Bacteria and Listeria, Switzerland (n = 195). The strains were of serotype 1/2a (n = 239), 1/2b (n = 44), 1/2c (n = 34), 3a (n = 5) and 4b (n = 70) and isolated between the years 1975 and 2013 (IV).

4.2 Typing (III, IV)

4.2.1 Serotyping (III, IV)

L. monocytogenes AL4E and AT3E and the 392 *L. monocytogenes* isolates were serotyped using the *Listeria* Antisera Set (Denka Seiken, Tokyo, Japan) including O- and H-factor antisera according to the manufacturer's instructions (III, IV) and by using the Pasteur typing tool (http://bigsdb.pasteur.fr/perl/bigsdb/bigsdb.pl?db=pubmlst_listeria_seqdef_public accessed 13 July 2017) (III).

4.2.2 Multilocus sequence typing (III, IV)

MLSTs of *L. monocytogenes* AL4E and AT3E were obtained from whole-genome sequences by using the PasteurMLST typing tool (Haase *et al.*, 2014, Moura *et al.*, 2016) (III). MLSTs of BC^{r} *L. monocytogenes* strains were defined by sequencing the PCR products of the seven housekeeping genes appointed to MLST typing (Ragon *et al.*, 2008) (IV).

Table 3. Bacterial strains and plasmids used in studies I–III.

Strain or plasmid	Genotype or characteristic [a]	Reference or source [b]
Listeria monocytogenes		
EGD-e	Wild-type strain, serotype 1/2a	(Glaser *et al*., 2001)
AL4E	Wild-type strain, serotype 1/2c	(Lundén *et al*., 2008)
AT3E	Wild-type strain, serotype 1/2c	(Lundén *et al*., 2008)
10403S	Wild-type strain, serotype 1/2a, streptomycin-resistant	(Bishop and Hinrichs, 1987)
Δ*lmo0050*	In-frame deletion of the *lmo0050* gene (1296 bp) with 1101 nucleotides	I, II
Δ*lmo0288* (Δ*yycG*)	Disruption of the wild type with 1581 bp insertion including out-of-frame deletion of the *yycG* gene (1833 bp) with 1682 nucleotides	I, II
Δ*lmo0692* (Δ*cheA*)	In-frame deletion of the *cheA* gene (1857 bp) with 1752 nucleotides	I, II
Δ*lmo1021*	In-frame deletion of the *lmo1021* gene (1059 bp) with 930 nucleotides	I, II
Δ*lmo1061*	Out-of-frame deletion of the *lmo1061* gene (1446 bp) with 1249 nucleotides	I, II
Δ*lmo1173*	Out-of-frame deletion of the *lmo1173* gene (1458 bp) with 1078 nucleotides	I, II
Δ*lmo1378* (Δ*lisK*)	In-frame deletion of the *lisK* gene (1452 bp) with 1116 nucleotides	I, II
Δ*lmo1508*	In-frame deletion of the *lmo1508* gene (1440 bp) with 1230 nucleotides	I, II
Δ*lmo1741*	In-frame deletion of the *lmo1741* gene (1041 bp) with 786 nucleotides	I, II
Δ*lmo1947* (Δ*resE*)	In-frame deletion of the *resE* gene (1791 bp) with 1542 nucleotides	I, II
Δ*lmo2011*	In-frame deletion of the *lmo2011* gene (1740 bp) with 1437 nucleotides	I, II
Δ*lmo2421* (Δ*cesK*)	In-frame deletion of the *cesK* gene (1143 bp) with 1020 nucleotides	I, II
Δ*lmo2500* (Δ*phoR*)	In-frame deletion of the *phoR* gene (1776 bp) with 1641 nucleotides	I, II
Δ*lmo2582*	Out-of-frame deletion of the *lmo2582* gene (1380 bp) with 1339 nucleotides	I, II
Δ*lmo2679* (Δ*kdpD*)	In-frame deletion of the *kdpD* gene (2691 bp) with 2652 nucleotides	I, II
Δ*yycG*c	Δ*yycG*, tRNA ^{Arg}::p*yycG*c, complemented strain	I
Δ*lisK*c	Δ*lisK*, tRNA ^{Arg}::p*lisK*c, complemented strain	I
Δ*agrC*c	Complemented strain, wild-type-like	II
Δ*liaS*c	Complemented strain, wild-type-like	II
Δ*virS*c	Complemented strain, wild-type-like	II
EGD-epPL2	EGD-e, tRNA ^{Arg}::pPL2	(Markkula *et al*., 2012b)

Table 3. *Continued*

Strain or plasmid	Genotype or characteristic [a]	Reference or source [b]
Δ*yycG*pPL2	Δ*yycG*, tRNA ^{Arg}::pPL2	I
Δ*lisK*pPL2	Δ*lisK*, tRNA ^{Arg}::pPL2	I
AT3Epc	AT3E strain, plasmid-cured	III
10403Spc*lpL*	10403S, tRNA ^{Arg}::p*clpL*	III
10403SpPL2	10403S, tRNA ^{Arg}::pPL2	III
Escherichia coli		
DH5α pMAD	DH5α strain containing the shuttle vector plasmid pMAD	(Mattila *et al*., 2011)
TOP10	Electrocompetent strain	Invitrogen
NEB5α	Electrocompetent strain	New England Biolabs
NEB5α	Chemically competent strain	New England Biolabs
HB101	Conjugation donor containing the helper plasmid pRK24	CRBIP
Plasmid		
pMAD	Cloning shuttle integration vector plasmid	(Arnaud *et al*., 2004)
pMAD- Δ*yycG*	pMAD containing homologous region up- and downstream of EGD-e *yycG*	I
pMAD- Δ*lisK*	pMAD containing homologous region up- and downstream of EGD-e *lisK*	I
pMAD- Δ*agrC*c	pMAD containing coding sequence of EGD-e *agrC*	II
pMAD- Δ*liaS*c	pMAD containing coding sequence of EGD-e *liaS*	II
pMAD- Δ*virS*c	pMAD containing coding sequence of EGD-e *virS*	II
pPL2	Site-specific integration vector	(Lauer *et al*., 2002)
p*yycG*c	pPL2 containing 613 bp upstream nucleotides and the coding sequence of *yycG*	I
p*lisK*c	pPL2 containing 1443 bp upstream nucleotides, the coding sequence and downstream terminator sequence of *lisK*	I
pLM58	Plasmid, AT3E strain	III
p*clpL*	pPL2 containing 423 bp of upstream nucleotides and the coding sequence of ATP-dependent protease *clpL*	III

[a] bp, base pairs; cds, coding sequence

[b] CRBIP, Biological Resource Centre of the Institut Pasteur

4.3 Growth conditions (I–IV)

All the strains were preserved in vials with beads (I–III) or in brain–heart infusion (BHI) broth (Oxoid, Cheshire, England) supplemented with 20% glycerol (Sigma-Aldrich, St. Louis, MO, USA) (IV) at –80 °C. *L. monocytogenes* strains and isolates were routinely grown at 37 °C on blood, on tryptic soy (TS) agar or in TS broth (Oxoid), on BHI agar or in BHI broth (BD, Franklin Lakes, NJ, USA and Oxoid), on Mueller-Hinton (MH) agar or in MH broth (Oxoid) or on ALOA (Harlequin™ Listeria Chromogenic) agar (Labema, Helsinki, Finland). *E. coli* strains were grown on Luria-Bertani (LB) agar or in LB broth (BD and Oxoid). Appropriate antibiotics

(Sigma-Aldrich), cadmium sulphate (3 $CdSO_4 \cdot 8\ H_2O$) (Merck, Darmstadt, Germany) and reserpine (Sigma-Aldrich) were added when needed.

4.4 Transcriptional analyses (I)

The transcription levels of each of the 15 HK-encoding genes in *L. monocytogenes* EGD-e were measured from total RNA extracted in the logarithmic growth phase (OD_{600}, 0.7) at 3 °C and at 30 min, 3 h and 7 h post-cold shock at 5 °C. Each experiment was repeated three times. Prior to the RNA extraction, the cells were lysed with 25 mg/ml lysozyme and 250 IU/ml mutanolysin (Sigma-Aldrich) in Tris-EDTA (Fluka Biochemica, Buchs, Switzerland) buffer in a 37 °C water bath for 30 min.

4.4.1 Total RNA extraction (I)

Total RNA was extracted by using the RNeasy Midi-kit (Qiagen, Venlo, the Netherlands). DNase treatment was performed by using the RNase-Free DNase set (Qiagen) and an additional DNase treatment by using the Ambion DNA-free kit (Ambion, Austin, TX, USA). The quantity of RNA was measured with a Nanodrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA), and RNA integrity was verified by using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA).

4.4.2 Real-time quantitative reverse transcription-PCR (I)

Reverse transcription from 800 ng of each total-RNA sample into complementary DNA (cDNA) was performed using the DyNAmo cDNA Synthesis Kit (Thermo Fischer Scientific, Inc., Waltham, MA, USA) in duplicate reactions. An additional predenaturation at 65 °C for 5 min and an elongated cDNA synthesis step at 40 °C for 40 min were used. A control reaction lacking the reverse transcriptase enzyme (no-RT control) was performed for each RNA sample. For real-time quantitative PCR (qPCR), each cDNA sample was diluted 1:1000. qPCR reactions were performed in duplicate in a RotorGene qPCR 3000 device (Corbett Research, Sydney, Australia) with the DyNAmo Flash SYBR Green qPCR Kit (Thermo Fischer Scientific, Inc.). The relative expression levels of the target HK-encoding genes in the logarithmic growth phase at 3 °C and at 30 min, 3 h and 7 h post-cold shock at 5 °C were calibrated to their expression levels in the logarithmic growth phase at 37 °C and to t_0 pre-shock, respectively, according to the Pfaffl method (Pfaffl, 2001). This method intrinsically includes the assay efficiencies, which for the target genes ranged from 0.88 to 1.00 and for the reference was 0.82 (Table 4). The 16S rRNA-encoding gene *rrn* was used as an internal normalization reference (Tasara and Stephan, 2007).

Table 4. Primers used in studies I–IV.

Application	Primer	Sequence (5' to 3') [a, b, c]	Reference
Real-time reverse transcriptase quantitative PCR			
	0050 F	AATCGCAGGTTTTGATGGA	I
	0050 R	AGTTCGTTGGTTGCCGTATT	I
	0288 F	AGCCCAAGCAATGACTGAAG	I
	0288 R	CGTGTCCCCGATGTCTAAAA	I
	0692 F	CGTCGTGAAAAATGCCAAG	I
	0692 R	CTAAAATCGTTGCCCCAGAA	I
	1021 F	ATGGCTGTTTGCTCAGGTTTA	I
	1021 R	GAAATGGCAGTAAGCGGAAC	I
	1061 F3	TCGTAAAAGCAGGCGAAGC	I
	1061 R3	TCGTGCCGTCTACAACAGTC	I
	1173 F	AAGAGGACGAGCAGGAATG	I
	1173 R	CGCAATAAGGAAACAACAGA	I
	1378 F	TGATGGGCAGAAGATGATGAC	I
	1378 R	GGAAGTGAGCGGATTTACCA	I
	1508 F	CTGCGGATGATAAGAAAAAG	I
	1508 R	AGCACGATAGAACCGACA	I
	1741 F	TCTCGTGCTGGGCTATTTCT	I
	1741 R	GTTTCTTCGCCATTAGTTGGT	I
	1947 F	GAACGGTGAAACTGGTGTTG	I
	1947 R	ATGCGAGAAGAAAAGACGAAAG	I
	2011 F2	CAAATGGGTTATCTGCTCCTTTAC	I
	2011 R2	TGTTTCGTTGGATGGTTGG	I
	2421 F2	CCCAGCAAGCCAGAAATG	I
	2421 R2	AAAATGGATGTAAGTGGTGTCC	I
	2500 F	TGGTAGAGGTGGACGAAG	I
	2500 R	AATAGTAACGGATGGGATTG	I
	2582 F	CAACTACTCACACTCGCTTCACTT	I
	2582 R	CCAACTCCACTCGGTCATTT	I
	2679 F	GCTAATGGGAGCGTTTGGT	I
	2679 R	TGTGTGAGCGGACCTTCTTC	I
	16S rRNA Forward	GATGCATAGCCGACCTGAGA	(Mattila *et al*., 2011)
	16S rRNA Reverse	CTCCGTCAGACTTTCGTCCA	(Mattila *et al*., 2011)
Mutant construction			
	0050-1	NNNNNNGGATCCTTATGGCTCAGACGGTATTCT	I, II
	0050-2	*TCATCCCCATTCTTCTACAT*GGCACTTACAAAAACAATCA	I, II
	0050-3	*TGATTGTTTTTGTAAGTGCC*ATGTAGAAGAATGGGGATGAA	I, II
	0050-4	NNNNNNACGCGTCCACACCTTTTGTCGTATCT	I, II
	0288-1 MluI	NNNNNNACGCGTCTTGTAGTAGATGACGAAAAACC	I, II
	0288-2	*CTAACCCTGTTCCTCCCATT*GTAAGCGAAAATACCCAAAA	I, II

Table 4. *Continued*

Application	Primer	Sequence (5' to 3') [a, b, c]	Reference
	0288-3	*TTTTGGGTATTTTCGCTTAC*AATGGGAGGAACAGGGTTAGG	I, II
	0288-4	NNNNNNACGCGTGCTTAGGAAAAGATTGCGTTTG	I, II
	0692-1	NNNNNNGGATCCCGTGAACAATCAATCCATCG	I, II
	0692-2	*CTGAAAAAGCCGCCAAGTGT*CATGCTATCCACCTCCATTTCT	I, II
	0692-3	*AAATGGAGGTGGATAGCATG*ACACTTGGCGGCTTTTTCAG	I, II
	0692-4	NNNNNNACGCGTGCCTGTTTCCCTTTTTCCTC	I, II
	1021-1 MluI	NNNNNNACGCGTCGTAAGGAGGTGTGTCAGC	I, II
	1021-2	*TTATTTTGATGGTGCCG*GAGCAAACAGCCATCATCAG	I, II
	1021-3	*CTGATGATGGCTGTTTGCTC*CGGCACCATCAAAATAA	I, II
	1021-4	NNNNNNACGCGTCACCAAATCGCCCAAGAC	I, II
	1061-1 MluI	NNNNNNACGCGTAAAAAGAACAGTAGGAGGCAAA	I, II
	1061-2	*ATAATAAACAACCCTAAACC*AGAGCCGTAAAAAGTAAAATGA	I, II
	1061-3	*ATTTTACTTTTTACGGCTCT*GGTTTAGGGTTGTTTATTATTGC	I, II
	1061-4	NNNNNNACGCGTCGCTGTATTTTCTTTGTCATCTG	I, II
	1173-1 MluI	NNNNNNACGCGTCGTGACAGGAATGAATGGAAG	I, II
	1173-2	*ATGACCGTTGTCCGTTACTT*TTTGGTTGTTTTTGGTGGTG	I, II
	1173-3	*CACCACCAAAAACAACCAAA*AAGTAACGGACAACGGTCAT	I, II
	1173-4	NNNNNNACGCGTATCAAACAGGGATAAGTTCG	I, II
	1378-1 MluI	NNNNNNACGCGTAGCGGTTGCTAATGATGGAC	I, II
	1378-2	*TCTGGCTTTATCTACACGGT*GCAGTTCTTTTACTTCTGGTTCTTCT	I, II
	1378-3	*ACCAGAAGTAAAAGAACTGC*ACCGTGTAGATAAAGCCAGAAGC	I, II
	1378-4	NNNNNNACGCGTCGAAAGAAGAACAAGCAACTATTC	I, II
	1508-1 MluI	NNNNNNACGCGTAATGTAAGAGGAGTGAGAACAGAA	I, II
	1508-2	*CCAATACCAGATTCGCCA*CAAGCAATGAGCAAAATGATAAA	I, II
	1508-3	*ATCATTTTGCTCATTGCTTG*TGGCGAATCTGGTATTGG	I, II
	1508-4	NNNNNNACGCGTACAGGGCGTCGTAAAGAAGT	I, II
	1741-1 MluI	NNNNNNACGCGTGGTCAAAAACAACCCCGATA	I, II
	1741-2	*CTCGTGCTGGGCTATTTCT*GTGGCGTAGGAACAGAAGTGA	I, II
	1741-3	*CACTTCTGTTCCTACGCCAC*AGAAATAGCCCAGCACGAGA	I, II
	1741-4	NNNNNNACGCGTAAGGCGGTAAGTTTGTTCCA	I, II
	1947-1 MluI	NNNNNNACGCGTGCCCCGAAGATGAACGAATA	I, II
	1947-2	*CAAAACAGCCAGTATCATCA*TGGAAGCACACAATGGAAAA	I, II
	1947-3	*TTTTCCATTGTGTGCTTCCA*TGATGATACTGGCTGTTTTGTCT	I, II
	1947-4	NNNNNNACGCGTTTTGTCGTGAACTGAGGGAGT	I, II
	2011-1 MluI	NNNNNNACGCGTGAAGTTTGTTCGGTTATTCCAA	I, II
	2011-2	*CAATCTCCAAAGAGCGGAA*GATACGAGCCACATCGGTCT	I, II
	2011-3	*AGACCGATGTGGCTCGTATC*TTCCGCTCTTTGGAGATTG	I, II
	2011-4	NNNNNNACGCGTGTGGGATTGTGTTTGGCTTT	I, II
	2421-1	NNNNNNGGATCCGAAAATCTCGCCTTGTTCA	I, II
	2421-2	*GTTTCTTGGGTTTTGGCGT*TTATCGTCAAACTGCCACTAAT	I, II
	2421-3	*TAGTGGCAGTTTGACGATAA*ACGCCAAAACCCAAGAAAC	I, II
	2421-4	NNNNNNGAATTCGACTGGAAAGGACAATGAGTATGA	I, II
	2500-1 MluI	NNNNNNACGCGTCATTCCGACAACCGCTACTC	I, II

Table 4. *Continued*

Application	Primer	Sequence (5' to 3') [a, b, c]	Reference
	2500-2	*TATGGCTGAAAATCGGGTT*TTCCATCGTCAAACATCTCG	I, II
	2500-3	*CGAGATGTTTGACGATGGAA*AACCCGATTTTCAGCCATA	I, II
	2500-4	NNNNNNACGCGTGCTTCTATTGTTACCTTGCTACA	I, II
	2582-1	NNNNNNACGCGTTCCCAGCCAACGAAAATAAC	I, II
	2582-2	*AGTTGTCACGATGCT*ATCCGATTCACATTCCGTTT	I, II
	2582-3	*AAACGGAATGTGAATCG*ACAAGCATCGTGACAACT	I, II
	2582-4	NNNNNNACGCGTGAGGCAAGTGATGGGGTAGA	I, II
	2679-1	NNNNNNACGCGTTTCTTCTGCCATCCGGTATC	I, II
	2679-2	*ATGGAAACGAATCGTCCAAGC*GGAGGAGATGGAAAATGAA	I, II
	2679-3	*TTCATTTTCCATCTCCTCCG*CTTGGACGATTCGTTTCCAT	I, II
	2679-4	NNNNNNACGCGTGCGTGCTTTATCCAGGTGTT	I, II
Complementation and conjugation			
	Komp 0288F1	NNNNGGATCCGGTGCGGATGACTATGTAACG	I
	Komp oper 0288R	NNNNTCTAGACCATTTTCTTTCCACTCAAACC	I
	Komp oper 1378F2	NNNNGGATCCTTTGCTCGTTACATTTCTGC	I
	Komp oper 1378R2	NNNNTCTAGATTATTCGTTTCCTTCACAGC	I
	BamHI *clpL* FN	NNNNGGATCCAGTTTCAAAGGTCGTTCTGGC	III
	BamHI *clpL* RN	NNNNGGATCCTCTATCAAGCAATCTCCTTCCC	III
	NC16	GTCAAAACATACGCTCTTATC	(Lauer *et al*., 2002)
	PL95	ACATAATCAGTCCAAAGTAGATGC	(Lauer *et al*., 2002)
Small-scale sequencing			
	Seq 0050 1F	ATGACAAGAATCGGACATTT	I
	Seq 0050 1R	TTTTTACATACCTTTTGCCTGA	I
	Seq 0050 2F	CGGAAAACACTAAAAAGAAAAGC	I
	Seq 0050 2R	CCGTTCTTCTGCTTGCTTC	I
	Seq 0288 1F	TTATGAAGGCAGGCCAGAAT	I
	Seq 0288 1R	GGAATAGTGCGGACGTGAAT	I
	Seq 0288 2F	CAGCCGAGGAAGAAGAAAAC	I
	Seq 0288 2R	GTGAAATAAACCGAATGTAGCC	I
	Seq 0692 1F	TTATCCGTTGCTGATGCTGA	I
	Seq 0692 1R	TGCGTTTTCCTCCCTTTTAC	I
	Seq 0692 2F	CGAGGGGCTTTTCTTTTGAT	I
	Seq 0692 2R	GACCTTTTCCCGTTTGGATAG	I
	Seq 1021 1F	GGAAGAAACATCCGACTAAAG	I
	Seq 1021 1R	CGCATTTTCATCCGTTG	I
	Seq 1021 2F	AAAGTTGGGCTTGTTGTTGC	I
	Seq 1021 2R	TTTTTCTGGTTTAGCGGTTAGC	I
	Seq 1061 1F	CGTTCGCTACTTGATGATG	I

Table 4. *Continued*

Application	Primer	Sequence (5' to 3') [a, b, c]	Reference
	Seq 1061 1R	CCAGGGAGCATCATTTG	I
	Seq 1061 2F	ATGCCGAGTATGGACGGGTA	I
	Seq 1061 2R	ACCTTTTGATGCGGAACCTT	I
	Seq 1173 1F	GCTCTTTTACTCTTTCGCTCAA	I
	Seq 1173 1R	TGATTTTTCTTGTTTCACGGTCTAC	I
	Seq 1173 2F	ACACGGAGAAAGCAAAAGGA	I
	Seq 1173 2R	ACAACAAAATCCCCAGCAAA	I
	Seq 1378 1F	TAACGAGTGGGTGCCTTTTT	I
	Seq 1378 1R	TACAGATGGGTTTTTCAGCA	I
	Seq 1378 2F	GCTAAACAAACAACGCTACAG	I
	Seq 1378 2R	CGCCCGAACAAAAGAAA	I
	Seq 1508 1F	TGGGGAGTTATGAAGAAAGTATGAA	I
	Seq 1508 1R	TCCAAGTTTTAGCACCAATGT	I
	Seq 1508 2F	TCCGTGAAGTAAGCGATGTG	I
	Seq 1508 2R	TGTGATTATGCCGATTGTCC	I
	Seq 1741 1F	AAGCAATAAACACGGCATCC	I
	Seq 1741 1R	TTGAAAAATGGAGAAGTAGTCGT	I
	Seq 1741 2F	AGTGTTGTTTTTGCCCCATC	I
	Seq 1741 2R	TTGTTGCTGTTGGCGTTAGT	I
	Seq 1947 1F	AACGAAAAGGTCTGCCAACA	I
	Seq 1947 1R	TGATTTGATGATGCCTGGTAA	I
	Seq 1947 2F	TTTACCATCCGCACCCAAT	I
	Seq 1947 2R	AGAATCAGCAGGAGGCACAC	I
	Seq 2011 1F	CGTTTTTGGTAGGCGATTAGAC	I
	Seq 2011 1R	CTTAGTATGGCTTTTCCTCGTATT	I
	Seq 2011 2F	GACGCTCTTCCCATTCTTCTT	I
	Seq 2011 2R	ACGGTGTTTGTCCACTTCCA	I
	Seq 2421 1F	ACAGTGCGGGACCATAATAAA	I
	Seq 2421 1R	GATGACGATTGAAAGTGCTCA	I
	Seq 2421 2F	GCATTTCCCTCCACCACTCT	I
	Seq 2421 2R	CAAGGCTGAAGACAACGACA	I
	Seq 2500 1F	TTTTCGTCTTTACCGCCAAC	I
	Seq 2500 1R	AACAGCACTTTATGAGAATGAATC	I
	Seq 2500 2F	TTTGTGCTTTAGGGTTAGTGTTTG	I
	Seq 2500 2R	CCAAGAGAAGTAGTGGCGAGA	I
	Seq 2582 1F	CATCCGAAACAGACAGCGTA	I
	Seq 2582 1R	TACGCTGTCTGTTTCGGATG	I
	Seq 2582 2F	AAAATTGGCAACCAAGAACG	I
	Seq 2582 2R	TCATCTGTGCCCACTTCAAA	I
	Seq 2679 1F	ATCAAGCGCTTCGAGTCAAT	I
	Seq 2679 1R	ACACGAATATCCCGAAGCAC	I
	Seq 2679 2F	TGCTGCGGCATATCAAGTAG	I
	Seq 2679 2R	AAACGTTTGGCCAATTTCAG	I

Table 4. *Continued*

Application	Primer	Sequence (5' to 3') [a, b, c]	Reference
Confirmation of plasmid curing			
	oriV-F	GAACAAGCGATCCGTCATGC	III
	oriV-R	TCGTTGCTAGGACTTGTCTGG	III
	clpL-F	ACAGGCTCGTGATGGCTTAC	III
	clpL-R	ACCGCGATATTGAGTTCCCG	III
Plate mating			
	ESAT-6 F	GCAATCAGTGGGAAGGACTG	III
	ESAT-6 R	ATCCATCGCTTGTTTTCCTG	III
	secA-F	ACTACTGCCAAAACATCGAAGC	III
	secA-R	AAGACGCACTGGATTCCCTC	III
Screening efflux pump systems			
	p1	CATTAGAAGCAGTCGCAAAGCA	(Elhanafi *et al*., 2010)
	p2	GTTTTCGTGTCAGCAGATCTTTGA	(Elhanafi *et al*., 2010)
	radC fwd	CTTGCCAATGATAATATCATC	(Müller *et al*., 2013)
	radC rev	GTGGTCTGAATGCTCCATCG	(Müller *et al*., 2013)
	EmrE fw	GACCAACACCACCTAAGT	IV
	EmrE rv	GTCTGATGGACTTACAAAGCT	IV

[a] N, any of the bases, i.e., adenine (A), cytosine (C), guanine (G) or thymine (T).

[b] Restriction sites are underlined.

[c] Overlapping 5'-end extensions of splicing-by-overlap extension PCR are marked in italics.

4.5 Genetic modifications (I–III)

In order to identify the role of HKs in the growth of *L. monocytogenes* EGD-e under different stresses, HK-encoding genes were deleted and restored (I, II). To assess the role of plasmid-borne ClpL protease in the heat resistance of *L. monocytogenes*, the plasmid-carrying AT3E strain was cured and the ClpL protease-encoding *clpL* gene introduced into heat-sensitive *L. monocytogenes* 10403S (III).

4.5.1 Knock-out deletion (I, II)

HK-encoding genes were individually deleted in *L. monocytogenes* EGD-e by allelic replacement using a pMAD shuttle vector (Arnaud *et al*., 2004), resulting in mutants without an associated antibiotic resistance gene. Target gene-deficient inserts with 700 to 800 bp up- and downstream regions were built by using splicing-by-overlap extension (SOE) PCR (Warrens *et al*., 1997). To construct the vector pMADΔ*target_gene*, the inserts were

individually ligated between the *Mlu*I and *Bam*HI or *Eco*RI sites of pMAD using T4 ligase (Thermo Fischer Scientific, Inc.). Propagated vectors were then separately electroporated (25 μF, 200 Ω, 2.3 kV) into *L. monocytogenes* EGD-e. Single-crossover mutants were selected at 39 °C with erythromycin (5 μg/ml) and double-crossovers without antibiotic. Deletions were confirmed by site-specific PCR and by sequencing the target regions (Institute of Biotechnology, University of Helsinki, Finland). Considering the putatively essential *yycG* gene, the mutational approach resulted in the disruption of the wild-type sequence of the *lmo0288–lmo0290* operon with the target gene-deficient insert, while harbouring the *yycG* coding sequence upstream of the insert. The primers used in mutant construction, PCR and small-scale sequencing are presented in Table 4.

4.5.2 Complementation (I, II)

To verify stress-sensitive mutant phenotypes, the coding sequences of the target gene wild-type copy with the related upstream regions, including the putative promoters (I, II), and related genes in putative operons (Toledo-Arana *et al.*, 2009) (I) were transformed into the respective phenotypically affected mutant strains. Complementation was performed by using the PSA prophage site-specific integration vector pPL2, provided by Prof. Martin Loessner (Swiss Federal Institute of Technology, Zürich, Switzerland) according to Lauer *et al.* (2002) (I), or by allelic replacement without an integrated plasmid using the pMAD vector (Arnaud *et al.*, 2004) (II). The amplified inserts were restricted with *Xba*I and *Bam*HI and ligated into the compatible *Spe*I and *Bam*HI sites of pPL2. The resultant plasmids were then conjugated into recipient *L. monocytogenes* strains according to Ma *et al.* (2011), and the strains carrying the pPL2 constructs were selected by chloramphenicol (25 μg/ml) on ALOA agar. Integration of pPL2 was verified by PCR using primers NC16 and PL95 (Lauer *et al.*, 2002) and the presence of the insert by using gene-specific primers (Table 4) (I). When complementation was performed by allelic replacement, inserts were amplified using the outermost primers for mutant strain construction (Table 4), ligated into the *Mlu*I site of pMAD, and transformed into recipient mutant strains according to Markkula *et al.* (2012b). Successful complementation was confirmed by PCR using gene-specific and outermost mutant construction primers (Table 4) (II).

4.5.3 Plasmid curing (III)

pLM58 plasmid curing in the heat-resistant *L. monocytogenes* AT3E was conducted using ten consecutive cultures at 40 °C for 24 h per incubation in TSB supplemented with 0.2 μg/ml novobiocin (Margolles and de los Reyes-Gavilán, 1998). Inoculations (1:100) into fresh broth were conducted between each incubation and final cultures were plated on TSA containing

novobiocin. Removal of the plasmid was confirmed from purified colonies by PCR with the pLM58-specific primers *oriV*-F and *oriV*-R and *clpL*-F and *clpL*-R (Table 4).

4.5.4 Plate mating (III)

To examine whether pLM58, harboured by *L. monocytogenes* AT3E, is self-transmissible, standard plate mating was conducted between *L. monocytogenes* wild-type AT3E and 10403S. The plasmid-borne *cadAC* genes, also found in pLM58, confer cadmium resistance (McLauchlin *et al.*, 1997, Lebrun *et al.*, 1994), which enables the exclusion of recipient cells that have not received pLM58. The innate streptomycin resistance of 10403S allows the selection of transconjugants from the donor (den Bakker *et al.*, 2012). Streptomycin-resistant 10403S, received from Prof. Martin Wiedmann, Cornell University, Ithaca, NY, USA, was routinely grown in BHI supplemented with 200 μg/ml streptomycin. Equal volumes (100 μl) of donor and recipient cells, grown to the logarithmic growth phase (OD_{600}, 0.5), were spotted on top of each other on BHI agar and incubated at room temperature for 1 h followed by incubation at 37 °C for 24 h. Transconjugants were screened on BHI containing 200 μg/ml streptomycin and 65 μg/ml or 130 μg/ml cadmium sulphate ($CdSO_4$). Colonies were screened by PCR using pLM58-specific *oriV* primers, and genomic AT3E ESAT-6 and 10403S *secA* primers (Table 4). To serve as both positive and negative controls, AT3E and 10403S were separately plated on BHI agar containing 130 μg/ml $CdSO_4$ or 200 μg/ml streptomycin.

4.5.5 Introducing *clpL* into a heat-sensitive *L. monocytogenes* strain (III)

To verify that *clpL* plays a role in the heat resistance of *L. monocytogenes*, the 423-bp upstream region including the putative promoter, and the coding sequence of *clpL* were amplified using primers BamHI *clpL* FN and BamHI *clpL* RN (Table 4) and conjugated into the heat-sensitive *L. monocytogenes* 10403S by filter mating using the integration vector pPL2 as described above (Lauer *et al.*, 2002, Ma *et al.*, 2011). Control pPL2 lacking the insert was separately conjugated into the recipient 10403S. Integration of the plasmids was confirmed by using NC16 and PL95 (Lauer *et al.*, 2002) and the presence of the insert by using *clpL* gene-specific primers (Table 4).

4.6 Phenotypic characterisation of *L. monocytogenes* strains and isolates (I–IV)

4.6.1 Growth curve analyses (I–III)

To analyse the growth of the *L. monocytogenes* wild-type and genetically modified strains exposed to cold, heat, osmotic, acid, alkali, ethanol or oxidative conditions, three to five separate colonies per strain were inoculated into 10 ml of BHI broth and incubated overnight at 37 °C (I–III). The cultures were diluted (1:100) in BHI broth (I–III) or BHI supplemented with 6% NaCl, 3.5 vol% ethanol or 5 mM H_2O_2, or BHI adjusted to pH 5.6 with 37% HCl or pH 9.4 with 5 M NaOH (II). Each dilution (300–350 µl) was transferred into separate wells of a 100-well honeycomb plate in duplicate technical replicates. The strains were grown in a Bioscreen C Microbiology Reader (Growth Curves Ltd., Helsinki, Finland) at 3 °C for 23 to 40 days (cold), at 42.5 °C or 42 °C for 10 to 24 h (heat), and at 37 °C for 10 to 48 h (other stresses). OD_{600} values were measured at 15-min or 1-h intervals. Control growths were performed at 37 °C for 24 h (I, II). The mean growth parameters, including the maximum growth rate, onset time of growth (lag phase) and maximum optical density, were obtained by using the DMFit web edition software (Computational Microbiology Research Group, Institute of Food Research, Colney, Norwich, UK) with the complete Baranyi and Roberts model (Baranyi and Roberts, 1994) (I, II), or by using the 'grofit' package (Kahm *et al.*, 2010) in R versions 3.2.2 and 3.4.0 (R Core Team, Vienna, Austria) applying the logistic model with the default settings (III). The area under the curve (AUC) was determined in 'grofit' (III) or by using a designed script in Matlab R2014a (MathWorks, Natick, MA, USA) (II). The correspondence between the OD_{600} values and the viable cell numbers of the wild-type and genetically modified strains was confirmed by plate counts in early and late logarithmic and early stationary growth phases (I, II), or mid-logarithmic and stationary growth phases (III).

4.6.2 Heat resistance assay (III)

L. monocytogenes strains 10403S, AL4E and AT3E, plasmid-cured AT3Epc, and conjugation strains 10403SpPL2 and 10403SpclpL were tested for heat survival according to Lundén *et al.* (2008), except that three overnight cultures of each strain were separately inoculated into TSB and challenged at 55 °C for 40 min. The decrease in the cell concentration (log_{10} reduction) was determined by colony counts on plate count (PCA) agar (Oxoid). Strains with a reduction of <1.0 log_{10} were considered heat resistant.

4.6.3 Minimum and maximum growth temperatures (I–III)

The differences in minimum and maximum growth temperatures were examined using the Gradiplate W10 incubator (BCDE Group, Helsinki, Finland) (Hinderink *et al.*, 2009). Dilutions of two (I, II) and three (III) separate overnight cultures of each strain were plated in duplicate by the stamping technique as parallel lines onto TSA (25 g agar/l). Minimum temperatures were measured for 21 days with a temperature gradient set to range from 1 °C to 7 °C (I). Differences in maximum growth temperatures were measured with a temperature gradient of 39 °C to 44 °C for 48 h (II) or 39.2 °C to 45.7 °C for 24 h (III). Growth boundaries were determined by using a stereomicroscope (Olympus SZ61, Nikon, Tokyo, Japan). The growth temperature threshold was determined as the boundary where dense bacterial growth discontinued (I–III).

4.6.4 Benzalkonium chloride resistance and impact of reserpine (IV)

The susceptibility of *L. monocytogenes* isolates to BC was tested using the agar dilution method (Elhanafi *et al.*, 2010). Single colonies of each strain were suspended into 100 µl of MH broth (Oxoid), of which 5 µl was spotted in technical duplicates onto MH agar (Oxoid) supplemented with 0 to 30 µg/ml BC. MICs were measured after 48 h at 37 °C. Strains displaying confluent growth in ≥20 µg/ml were considered BC resistant (BC^r).

Efflux pump activity in BC^r *L. monocytogenes* isolates was assessed by measuring the BC MICs on MH agar (Oxoid) supplemented with 0 µg/ml to 30 µg/ml BC and an additional 20 µg/ml of the efflux pump inhibitor reserpine (Sigma-Aldrich). The BC resistance of the *L. monocytogenes* isolates was categorised as fully efflux pump-dependent when reserpine caused a ≥10 µg/ml decrease in the BC MIC and partially dependent when the resultant decrease was <10 µg/ml.

4.7 Genomic analyses (III)

4.7.1 DNA extraction (III)

After growth to the stationary growth phase at 37 °C, the cells of the *L. monocytogenes* AT3E and AL4E strains were lysed using lysozyme and mutanolysin at 37 °C for 2 h. Genomic DNA was extracted by using guanidium thiocyanate (Pitcher *et al.*, 1989). DNA integrity was verified by gel electrophoresis and the DNA yield was measured by using the Qubit dsDNA BR Assay Kit (Life Technologies, Carlsbad, CA, USA).

4.7.2 Sequencing (III)

WGS of *L. monocytogenes* AL4E and AT3E was conducted by the Institute of Biotechnology (Helsinki, Finland) using single-molecule real-time (SMRT) sequencing in the PacBio RS platform with coverages of 199x (AL4E), 252x (AT3E) and 422x (pLM58) (III). AL4E and AT3E genomes were *de novo* assembled according to the RS_HGAP_Assembly 3 protocol (Pacific Biosciences of California, Inc., Menlo Park, CA, USA).

4.7.3 Annotation and comparative genomic analysis (III)

L. monocytogenes AL4E and AT3E genomes were annotated by using RAST 2.0 (Aziz *et al.*, 2008) and genome comparison was conducted in SEED Viewer 2.0 (Overbeek *et al.*, 2005). Sequence-based operon prediction of pLM58 was performed by using FGENESB (Solovyev and Salamov, 2011) and putative prophages of pLM58 were predicted by using PHASTER (Zhou *et al.*, 2011, Arndt *et al.*, 2016). Circular visualisation and comparison of AL4E and AT3E chromosomes was conducted by using BRIG (BLAST Ring Image Generator) (Alikhan *et al.*, 2011) and pLM58 was visualized by using SnapGene Viewer 3.3.4 (GSL Biotech LLC, Chicago, IL). The coding sequences of the replication initiation protein and the ATP-dependent protease ClpL harboured by pLM58 were compared using BLASTN 2.2.26 (Altschul *et al.*, 1997) to *L. monocytogenes* plasmid sequences deposited in GenBank at NCBI by 13 July 2017.

4.8 Statistical analyses (I–IV)

The statistical analyses were performed in Excel 2010 (Microsoft, Redmond, WA, USA) (I), in IBM SPSS Statistics 23 and 24 (IBM, Armonk, NY, USA) (II, III) and using the JMP program v. 11.0.0 (SAS Institute, Inc., NC, USA) (IV). Differences in the expression levels of the HK-encoding genes were tested by the paired *t*-test (I). Differences in the growth parameters (I–III), the minimum and maximum growth temperatures (I–III) and the $\log_{10}$ reductions (III) were tested using the independent-samples two-tailed *t*-test. Fisher's exact test was used in pairwise comparisons of BC resistance and sensitivity within serotypes and isolate origin (IV).

5 RESULTS

5.1 Roles of two-component-system histidine kinases in the stress tolerance of *L. monocytogenes* (I, II)

5.1.1 Relative expression levels of histidine kinase-encoding genes at low temperatures (I)

At 3 °C, *cheA* (*lmo0692*) was upregulated 236-fold ($P < 0.001$) as compared to the expression level at 37 °C, while *liaS* (*lmo1021*), *lmo1173*, *lisK* (*lmo1378*), *lmo1508*, *lmo1741*, *resE* (*lmo1947*) and *cesK* (*lmo2421*) displayed 1.6- to 3-fold ($P < 0.05$) upregulation (Fig. 2). At each time point post-cold shock from 37 °C to 5 °C, *lmo0050*, *yycG* (*lmo0288*), *lmo1061*, *lmo1741* and *kdpD* (*lmo2679*) were upregulated 1.9- to 3.4-fold ($P < 0.05$) as compared to t_0 (Figs 3A–C). *liaS* and *phoR* (*lmo2500*) were upregulated 1.5- to 2.1-fold ($P < 0.05$) both at 30 min and 3 h (Figs 3A and B). Cold shock induced the expression of *cheA* 7.8-fold ($P < 0.01$) at 7 h (Fig. 3C), and that of *lmo2011* 1.6-fold ($P < 0.05$) at 30 min (Fig. 3A). The expression of other HK-encoding genes was unaffected under cold stress.

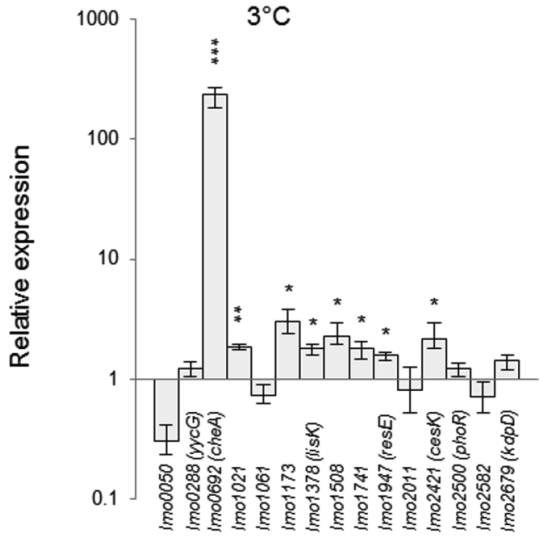

Figure 2 Relative gene expression levels of the HK-encoding genes in *L. monocytogenes* EGD-e during the logarithmic growth phase at 3 °C in relation to the expression level in logarithmic growth at 37 °C. Gene expression was normalized to *16S rrn*. Error bars represent the minimum and maximum ratios between three replicate cultures. Significantly increased relative expression levels ($P < 0.05$, < 0.01 or < 0.001, paired *t*-test) are indicated by asterisks *, ** and ***, respectively (I).

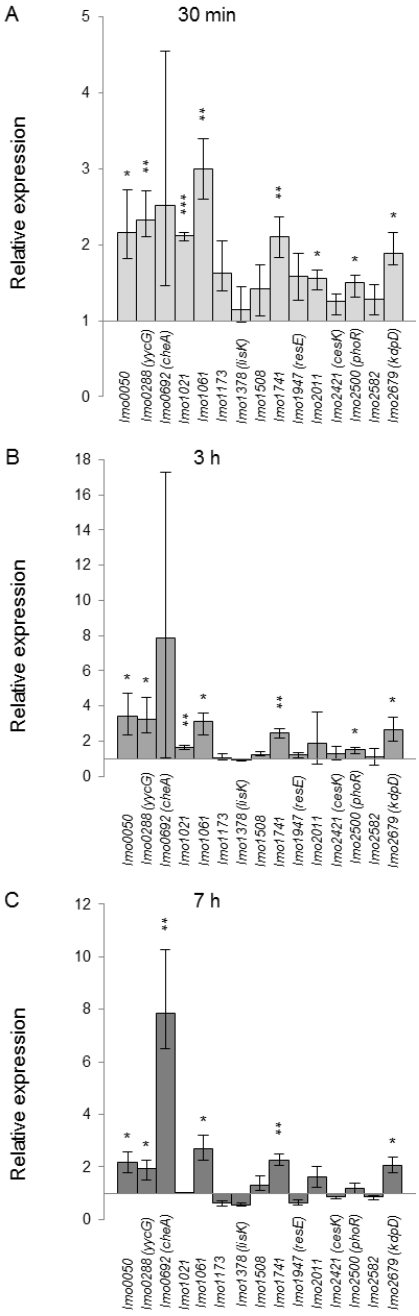

Figure 3 Relative expression levels of the HK-encoding genes in *L. monocytogenes* EGD-e 30 min (A), 3 h (B) and 7 h (C) post-cold shock from 37 °C to 5 °C, each calibrated to t_0 at 37 °C. Gene expression was normalized to *16S rrn*. Error bars represent the minimum and maximum ratios between three replicate cultures. Significantly increased relative expression levels ($P < 0.05$, < 0.01 or < 0.001, paired *t*-test) are indicated by asterisks *, ** and ***, respectively (I).

5.1.2 Growth of genetically modified *L. monocytogenes* under various stresses (I, II)

At 3 °C, the growth of the Δ*lisK* deletion mutant strain was nearly completely restricted, with the growth rate 86% ($P < 0.001$) lower and the maximum optical density 73% ($P < 0.001$) lower than those of the wild-type EGD-e strain. Δ*yycG* displayed a prolonged lag phase, a 31% ($P < 0.001$) lower growth rate and an 8% ($P < 0.01$) lower maximum optical density compared to the wild type. Δ*resE* displayed a later onset of growth and a 29% ($P < 0.001$) lower growth rate than those of the wild type (I). Complementation of Δ*yycG* restored the cold-sensitive phenotype to the wild-type level, while complementation of Δ*lisK* did not fully restore growth at low temperature, although a slight increase in the maximum optical density was observed (I).

The growth of Δ*liaS* was impaired under each stress condition other than low temperature when compared to EGD-e, with marked changes at high temperature (42.5 °C) and under osmotic stress (6% NaCl), moderate changes under pH stresses (5.6 and 9.4) and with minor changes in 3.5% ethanol and 5mM H_2O_2. AUC values of Δ*liaS* were 31% and 30% lower ($P < 0.01$) under osmotic and heat stresses, respectively, 21% and 15% lower ($P < 0.001$) under acid and alkali stresses, respectively, and 11% lower ($P < 0.01$) under both ethanol and oxidative stresses compared to AUC values of EGD-e under corresponding stress conditions. The maximum growth rates of Δ*liaS* decreased by 67% ($P < 0.001$) in 6% NaCl and 15% to 46% ($P < 0.01$) under other stresses. Δ*virS* displayed almost completely restricted growth at 42.5 °C and moderately impaired growth in ethanol. The AUC values of Δ*virS* were 58% and 19% lower ($P < 0.01$) under heat and ethanol stresses, respectively. The maximum growth rate of Δ*virS* in ethanol was 33% lower ($P < 0.01$), while its growth rate at high temperature was negligible. The Δ*agrC* strain displayed notably impaired growth under osmotic stress, with a 33% lower ($P < 0.01$) maximum growth rate. Δ*agrC* also displayed slight growth deficiency and a 14% lower ($P < 0.01$) AUC value under oxidative stress (II). Complementation restored the wild-type phenotypes to the Δ*agrC*, Δ*liaS* and Δ*virS* mutants (II). At 37 °C, the growth of none of the mutant strains deviated from that of the wild-type EGD-e (I, II).

Table 5. Mean maximum growth rates of *L. monocytogenes* wild-type EGD-e and HK mutant strains. Modified from I and II.

	Maximum growth rate ± SD (OD_{600} units/h) [a]						
Strain	4 °C	42.5 °C	pH 5.6	pH 9.4	6% NaCl	3.5% ethanol	5 mM H_2O_2
EGD-e	0.0035 ± 0.0001	0.059 ± 0.016	0.068 ± 0.001	0.13 ± 0.004	0.006 ± 0.0000	0.06 ± 0.006	0.22 ± 0.007
Δ*lmo0050* (Δ*agrC*)	0.0057 ± 0.0005	0.068 ± 0.030	0.072 ± 0.001	0.12 ± 0.003*	0.004 ± 0.0000*	0.05 ± 0.005	0.18 ± 0.004
Δ*lmo0288* (Δ*yycG*)	0.0024 ± 0.0003**	0.065 ± 0.013	0.062 ± 0.001*	0.20 ± 0.011	0.007 ± 0.0000	0.04 ± 0.003	0.23 ± 0.008
Δ*lmo0692* (Δ*cheA*)	0.0033 ± 0.0001	0.056 ± 0.015	0.063 ± 0.001**	0.12 ± 0.003	0.006 ± 0.0000	0.05 ± 0.004	0.21 ± 0.006
Δ*lmo1021* (Δ*liaS*)	0.0055 ± 0.0007	0.050 ± 0.015	0.043 ± 0.001**	0.07 ± 0.002**	0.002 ± 0.0000**	0.04 ± 0.003*	0.17 ± 0.004**
Δ*lmo1061*	0.0050 ± 0.0004	0.060 ± 0.014	0.062 ± 0.001**	0.13 ± 0.004	0.006 ± 0.0000	0.05 ± 0.004	0.22 ± 0.007
Δ*lmo1173*	0.0044 ± 0.0003	0.058 ± 0.014	0.062 ± 0.001*	0.13 ± 0.004	0.008 ± 0.0001	0.05 ± 0.005	0.22 ± 0.007
Δ*lmo1378* (Δ*lisK*)	0.0005 ± 0.0003**	0.058 ± 0.023	0.065 ± 0.001	0.16 ± 0.005	0.009 ± 0.0001	0.07 ± 0.008	0.22 ± 0.007
Δ*lmo1508*	0.0056 ± 0.0005	0.062 ± 0.014	0.063 ± 0.001*	0.24 ± 0.009	0.008 ± 0.0001	0.06 ± 0.005	0.22 ± 0.008
Δ*lmo1741* (Δ*virS*)	0.0044 ± 0.0002	0.000 ± 0.003*	0.066 ± 0.002*	0.16 ± 0.006	0.007 ± 0.0000	0.04 ± 0.003*	0.24 ± 0.008
Δ*lmo1947* (Δ*resE*)	0.0025 ± 0.0001**	0.051 ± 0.014	0.061 ± 0.001*	0.13 ± 0.004	0.008 ± 0.0001	0.05 ± 0.004	0.21 ± 0.007
Δ*lmo2011*	0.0036 ± 0.0001	0.051 ± 0.010	0.065 ± 0.001*	0.13 ± 0.004	0.009 ± 0.0001	0.05 ± 0.005	0.22 ± 0.008
Δ*lmo2421* (Δ*cesK*)	0.0036 ± 0.0002	0.054 ± 0.015	0.065 ± 0.001	0.13 ± 0.005	0.009 ± 0.0001	0.06 ± 0.009	0.21 ± 0.006
Δ*lmo2500* (Δ*phoR*)	0.0032 ± 0.0002	0.050 ± 0.013	0.064 ± 0.001	0.21 ± 0.007	0.009 ± 0.0002	0.05 ± 0.005	0.18 ± 0.004
Δ*lmo2582*	0.0040 ± 0.0002	0.073 ± 0.026	0.069 ± 0.001	0.12 ± 0.003*	0.007 ± 0.0001	0.05 ± 0.005	0.21 ± 0.006
Δ*lmo2679* (Δ*kdpD*)	0.0079 ± 0.0007	0.064 ± 0.025	0.068 ± 0.001	0.13 ± 0.004	0.006 ± 0.0000	0.05 ± 0.004	0.22 ± 0.007

[a] Significantly decreased values ($P < 0.01$, $P < 0.001$, 2-tailed *t*-test) compared to the corresponding value of the wild type are indicated by asterisks * and **, respectively.

5.1.3 Minimum and maximum growth temperatures (I, II)

The minimum growth temperatures of Δ*yycG* and Δ*lisK* were 3.7 °C and 2.7 °C higher (P < 0.001), respectively, when compared to EGD-e after a 21-day incubation, while Δ*cesK* and Δ*kdpD* presented 2.3 °C and 0.5 °C (P < 0.05) higher minimum growth temperatures, respectively (I). The maximum growth temperature of Δ*liaS* was 0.9 °C lower (P < 0.001) and that of Δ*virS* 0.7°C lower (P < 0.001) than EGD-e (Fig. 4) (II).

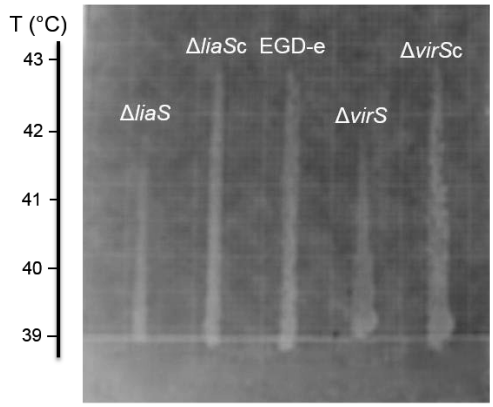

Figure 4 Maximum growth temperatures of *L. monocytogenes* EGD-e, the histidine kinase deletion mutant strains Δ*liaS* and Δ*virS* and the complemented strains Δ*liaS*c and Δ*virS*c within 48 h in the Gradiplate W10 incubator in a temperature gradient of 39 °C to 44 °C (II).

5.2 Plasmid-mediated heat resistance in *L. monocytogenes* (III)

5.2.1 Novel 58-kb plasmid contributes to the heat resistance of *L. monocytogenes* AT3E (III)

Genome analysis revealed the heat-resistant AT3E to harbour a novel plasmid pLM58 (Fig. 5). The 58-kb plasmid, with a GC content of 36.6%, contains 70 predicted ORFs and 19 predicted operons. Plasmid curing resulted in significantly impaired heat resistance of the derivative AT3Epc compared to the AT3E parent, with a $\log_{10}$ reduction of 1.1 cfu/ml (P < 0.001) at 55 °C (Fig. 6).

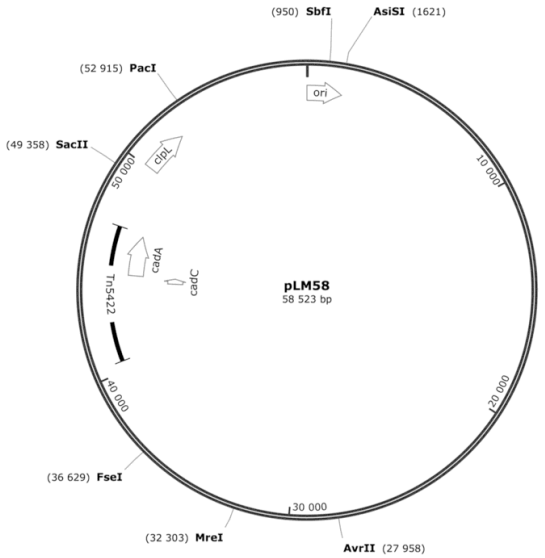

Figure 5 Genetic map of *L. monocytogenes* plasmid pLM58. Initiation of replication protein-encoding *ori*, ATP-dependent protease-encoding *clpL*, cadmium resistance genes *cadA* and *cadC*, transposon Tn*5422* and unique restriction sites are indicated (III).

5.2.2 Plasmid-borne *clpL* increases heat resistance in *L. monocytogenes* (III)

Annotation of pLM58 revealed an ORF putatively encoding an ATP-dependent ClpL protease (Fig. 5). Introduction of *clpL* augmented the heat resistance of natively heat-sensitive *L. monocytogenes* 10403S, observed by a significant decrease in $\log_{10}$ reduction from 1.2 cfu/ml to 0.4 cfu/ml ($P < 0.01$) at 55 °C (Fig. 6).

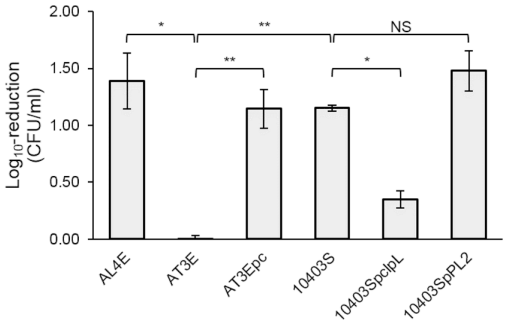

Figure 6 pLM58 plasmid-borne ATP-dependent protease ClpL mediates heat resistance in *L. monocytogenes*. Error bars represent the standard deviations of three replicate cultures. Statistically significant $\log_{10}$ reduction values ($P < 0.01$, $P < 0.001$, independent samples 2-tailed *t*-test) are indicated by asterisks * and **, respectively. NS, not significant. (III)

5.2.3 pLM58 is putatively nonconjugative (III)

Upon standard plate mating between AT3E and 10403S, no colonies were detected on selective plates containing both 130 μg/ml $CdSO_4$ and 200 μg/ml streptomycin, indicating no conjugative transmission of pLM58. pLM58 also lacked the known T4SS-encoding genes required for the conjugation of self-transmissible plasmids.

5.3 *L. monocytogenes* strains display high variability under severe stresses (III, IV)

5.3.1 Resistance and growth of *L. monocytogenes* strains vary at high temperatures (III)

We first tested the heat resistance at 55 °C, growth at 42 °C and maximum growth temperature of *L. monocytogenes* AL4E and AT3E in order to elucidate the differences between their thermoresistance and growth at high temperature. With a 0.0 cfu/ml $\log_{10}$ reduction, *L. monocytogenes* AT3E proved to be more heat resistant than AL4E (1.4 cfu/ml $\log_{10}$ reduction; $P < 0.01$) at 55 °C. At 42°C, the differences between their growth were negligible. Strain AL4E exhibited a 0.5 °C higher maximum growth temperature than the heat-resistant AT3E strain ($P < 0.01$).

5.3.2 *L. monocytogenes* isolates display variable resistance to benzalkonium chloride (IV)

The BC resistance profiles for the Finnish and Swiss *L. monocytogenes* isolates were determined. BC MICs ranged from 7.5 μg/ml to over 30 μg/ml. Isolates with BC MIC of ≥ 20 μg/ml were considered BC^r. Altogether, 11.5% (45/392) of the strains proved to be resistant to BC. Considering the isolation country, 10.7% (21/107) and 12.3% (24/195) of isolates of Finnish and Swiss origin strains, respectively, were BC^r.

Overall, most of the BC^r strains belonged to lineage II (39/45; 86.7%). Considering serotypes, the highest prevalence of BC^r strains was found among serotype 1/2c (11/34; 32.4%), while, in total, most of the resistant strains belonged to the serotype 1/2a (28/45, 62.2%). Genotypically, the 45 BC^r strains belonged to 14 MLSTs, of which two were the newly described ST25 and ST28. Most of the BC^r strains were of ST121 (14/45; 31.1%) and ST9 (11/45; 24.4%). Furthermore, most of the BC^r strains were associated with clonal complexes (CC) CC121 (16/45; 35.6%) and CC9 (11/45; 24.4%).

PCR-based analysis revealed 68.9% (31/45) of the BC^r strains to harbour at least one of the known BC resistance-conferring genes *bcrABC*, *emrE* or *qacH*. *bcrABC* was found in Swiss isolates alone, while only one BC^r isolate, that of Finnish origin, was found to harbour *emrE*. A screen with reserpine

showed that 40% (18/45) of the BC^r strains were at least partially efflux pump-dependent. Of the 14 strains lacking the known BC resistance-conferring genes, the BC resistance of three strains proved to be independent of efflux pump systems.

6 DISCUSSION

6.1 Extensive roles of two-component-system histidine kinases in the stress tolerance of *L. monocytogenes* (I–II)

In these studies we assessed the roles of HK-encoding genes in the stress tolerance of *L. monocytogenes* to ambient stress conditions, low and high temperatures, low and high pH levels, and high salt, ethanol and hydrogen peroxide concentrations, widely used to control bacterial contamination in food processing and preservation (NicAogáin and O'Byrne, 2016, Bucur *et al.*, 2018). While RRs of *L. monocytogenes* have been more extensively studied, information has been lacking on the role of HKs. However, phosphorylation of the HK is a key step in the phosphotransfer chain that further governs the phosphorylation of the cognate RR and the final output (Stock *et al.*, 2000, Jacob-Dubuisson *et al.*, 2018), and the functions of HKs are thus of special interest.

Of the HKs, YycG and LisK proved to be essential to the cold tolerance of *L. monocytogenes*. Based on expressional analyses of HK-encoding genes, YycG seems to be particularly needed under abrupt cold stress, while the role of LisK is accentuated during cold acclimation. In addition, ResE appears to play a minor role in the early growth phase of *L. monocytogenes* under cold conditions. The contribution of all of these TCSs to cold tolerance might be explained by potential cell envelope-related stress responses (Mohedano *et al.*, 2005, Williams *et al.*, 2005a, Gottschalk *et al.*, 2008, Nielsen *et al.*, 2012). The YycFG system (synonymous for WalKR) is regarded as essential and is highly conserved among low G+C-content Gram-positive bacteria such as *B. subtilis* (Fabret and Hoch, 1998, Szurmant *et al.*, 2007, Takada *et al.*, 2018) and *S. aureus* (Martin *et al.*, 1999, Poupel *et al.*, 2016). Due its essential and conserved nature, YycFG has attracted long-lasting research interest as a potential antimicrobial target (Fabret and Hoch, 1998, Dubrac *et al.*, 2008, Tiwari *et al.*, 2017).

We concluded HK kinase LiaS to have a universal impact on the stress tolerance of *L. monocytogenes* EGD-e. Mutational studies revealed impaired growth of Δ*liaS* under each stress condition studied except cold: under heat, acid, alkali, osmotic, ethanol, and oxidative conditions. The most prominent roles were noted upon heat and osmotic stresses at 42.5 °C and in 6% NaCl, respectively. Since the upkeep of the cell wall structure and membrane functions have a key role in environmental stress adaptation (Soni *et al.*, 2011, NicAogáin and O'Byrne, 2016), the comprehensive role of the TCS LiaSR may be attributable to its actions of remodelling the cytoplasmic membrane composition in response to cell-envelope disturbances (Fritsch *et al.*, 2011).

Besides LiaSR-mediated stress responses, our studies, combined with previous findings on RRs YycF (Chan *et al.*, 2007b), LisR (Chan *et al.*, 2008), VirR (Williams *et al.*, 2005a) and AgrA (Garmyn *et al.*, 2012), point out that the complete TCSs YycFG and LisRK, AgrCA and VirRS play roles under cold, osmotic and ethanol stresses, respectively. The potential versatility in stress tolerance of the Agr quorum sensing system, which includes AgrCA, is illustrated by the advantage that it provides *L. monocytogenes* in survival in natural soil, the most complex growth environment (Vivant *et al.*, 2014, Vivant *et al.*, 2015). VirRS and the Agr quorum sensing system have also been associated with the virulence of *L. monocytogenes* (Autret *et al.*, 2003, Mandin *et al.*, 2005, Riedel *et al.*, 2009, de las Heras *et al.*, 2011, Grubaugh *et al.*, 2018), with VirR playing a key role as a virulence regulator and the *virR* gene itself being upregulated *in vivo* during infection in a mouse model (Camejo *et al.*, 2009). VirR potentially participates in virulence by gene regulation of cell-surface modulation (Mandin *et al.*, 2005, Camejo *et al.*, 2009). Alterations in cell-envelope structures were, in turn, suggested to result in enhanced ethanol tolerance (Williams *et al.*, 2005a). Furthermore, exposure to osmotic stress similar to conditions in the human intestine increases virulence gene expression in *L. monocytogenes* (Sue *et al.*, 2004). Due to these potential interfaces between virulence and stress tolerance mechanisms, it may not be surprising that both TCSs AgrCA and VirRS would be involved not only in virulence but also in stress tolerance in *L. monocytogenes*. However, it should be further noticed that, instead of the toxin production-controlling VirRS system, extensively described in *Clostridium perfringens* (Lyristis *et al.*, 1994, Ohtani *et al.*, 2010) in which the regulatory function occurs via RNA molecules (Shimizu *et al.*, 2002), VirRS of *L. monocytogenes* is a member of the BceRS-like TCSs (Dintner *et al.*, 2011).

Intriguingly, cognate TCS partners do not always seem to feature together in the same stress tolerance responses. Besides the obvious methodological differences, as well as potential variation within parental strains between studies (Bécavin *et al.*, 2014), cross-phosphorylation (cross-talk or cross-regulation) may take place between noncognate partners (Laub and Goulian, 2007, Siryaporn and Goulian, 2010). Indeed, while we found LiaS to play a significant role in heat stress, the same is evidently not the case with its cognate RR LiaR (Kallipolitis and Ingmer, 2001, Williams *et al.*, 2005a). LiaS is a potential phosphorylator of the orphan RR DegU (Williams *et al.*, 2005a), also assigned a role in contributing to the heat tolerance of *L. monocytogenes* (Gueriri *et al.*, 2008). Mandin *et al.* (2005) also suggested that, inside a host, VirR activation might be unrelated to the cognate HK VirS but occur via cross-talking or molecular compound phosphorylation instead. Verifying cross-phosphorylation between noncognate partners would require further investigations into protein–protein interactions between particular HKs and RRs (Bielecki *et al.*, 2015).

For those HK-encoding genes upregulated at low temperatures while presenting no significant changes in mutational studies, the apparent discrepancies may be due to putative posttranscriptional regulation. For instance, Csp-encoding genes are mainly regulated at the posttranscriptional level (Narberhaus *et al.*, 2006). Overall, we found that, apart from the chemotaxis-encoding *cheA*, the relative expression levels of the HK-encoding genes at low temperatures only moderately differed from that of the expression levels at 37 °C. Although a recent comparative transcriptome analysis demonstrated fairly high transcript levels (8.9-fold change) for HK-encoding *kdpD* upon sodium lactate adaptation (Suo *et al.*, 2018), as such, transcriptional studies may not be the optimum single method of choice to unambiguously ascertain the role of a certain HK in the stress tolerance of *L. monocytogenes*.

Although the presence and the output domain types of the TCSs of *L. monocytogenes* have been described (Glaser *et al.*, 2001, Williams *et al.*, 2005a), much remains to be discovered concerning the detailed structures and the triggering signals of the TCS signalling structures of *L. monocytogenes* (Zetzmann *et al.*, 2016, Pinheiro *et al.*, 2018). Furthermore, by collecting phenotypic data with a considerably larger number of strains than in the present studies, the discrepancies between the putative roles of certain TCSs reported in previous studies could possibly be elucidated. Strain and population heterogeneity might influence the results when using only one or few model strains, particularly when using laboratory strains that do not always optimally represent the naturally occurring *L. monocytogenes* strains (Bécavin *et al.*, 2014).

6.2 pLM58-borne ClpL-mediated heat resistance in *L. monocytogenes* (III)

This study is very plausibly the first description of plasmid-mediated heat resistance in *L. monocytogenes*, conferred via a novel plasmid, pLM58. Genes potentially related to heat tolerance have been annotated in *L. monocytogenes* plasmids (Fox *et al.*, 2016). Canchaya *et al.* (2010) also identified a *clpL* gene that was suggested to originate from lactic acid bacteria, in which *clpL* was upregulated during heat shock (Suokko *et al.*, 2005). However, conclusive evidence of plasmid-borne heat resistance-mediating genes in *L. monocytogenes* has been lacking to date. Our results indicate that plasmid-borne ClpL is a potential predictor of augmented heat resistance in *L. monocytogenes*. As heat treatment is a central means to control *L. monocytogenes* contamination during food processing (Bucur *et al.*, 2018), the possibility of a heat resistance-conferring trait harboured by a MGE in a foodborne pathogen raises serious concerns with regard to food hygiene and safety. Of other pathogenic bacteria, a heat resistance-mediating plasmid has previously been described at least in *Klebsiella pneumoniae*

(Bojer *et al.*, 2010, Bojer *et al.*, 2012). Regarding plasmid-mediated traits within bacteria, the focus of the past and present research has been on the rapidly emerging antibiotic resistance (Poyart-Salmeron *et al.*, 1990, Liu *et al.*, 2016, Liu *et al.*, 2019, Schweizer *et al.*, 2019, Slettemeås *et al.*, 2019), while much less in known about the plasmid-mediated environmental stress tolerance of pathogenic bacteria. However, the possible cotransfer of both stress tolerance and antibiotic resistance-conferring traits cannot be overlooked, as they may have grave implications for consumer and patient safety considering both foodborne and nosocomial infections. Notably, Bojer *et al.* (2012) detected the cotransfer of both multidrug and heat resistance-conferring traits in a conjugative plasmid in *K. pneumoniae*.

Instead of focusing on particular genes, our aim in this study was to comprehensively approach the subject of finding novel genetic traits conferring heat resistance in *L. monocytogenes*. Furthermore, we wanted to take into account the possibility of plasmids acting as heat-resistance mediators. Consequently, WGS and comparison of the complete genomes of a heat-resistant and heat-sensitive strain were opted for. Indeed, whole-genome comparison revealed the most prominent difference between the heat-resistant AT3E and heat-sensitive AL4E *L. monocytogenes* strains to be a novel 58-kb plasmid. Furthermore, plasmid curing generated a significant decrease in heat resistance of the AT3E strain at 55 °C. pLM58 harboured a 2,115-bp ATP-dependent protease-encoding *clpL* gene. Introducing this gene to a naturally heat-sensitive *L. monocytogenes* 10403S resulted, in turn, in significantly improved survival under heat challenge. While chromosomal stress resistance-mediating Clp ATPases have been described in *L. monocytogenes* (Rouquette *et al.*, 1996, Nair *et al.*, 2000), this is the first study to identify a plasmid-borne Clp in *L. monocytogenes* conferring heat resistance. As the same *clpL* gene can be found in other *Listeria* spp. plasmids sequenced to date, heat resistance may prove to be even more prevalent within plasmid-harbouring *L. monocytogenes* strains.

As plate mating resulted in no apparent transconjugants, it seems that pLM58 is not self-transmissible. This was supported by the absence of the known type IV secretion system-encoding genes (Smillie *et al.*, 2010, Goessweiner-Mohr *et al.*, 2014) in pLM58. Whether pLM58 is mobilisable needs to be further investigated. However, due to the mosaic nature of *L. monocytogenes* plasmids (Canchaya *et al.*, 2010, Schmitz-Esser *et al.*, 2015), the possibility of a conjugative plasmid harbouring a heat resistance-mediating *clpL* gene in *L. monocytogenes* still remains. As WGS methods become increasingly common and affordable and as complete sequences of new *Listeria* spp. plasmids become available in open databases, future comparative genomic analyses will undoubtedly enlighten the contents and potential heat resistance-mediating genes harboured by other, possibly conjugative plasmids.

Neither plasmid curing nor introducing the *clpL* into a heat-sensitive *L. monocytogenes* strain resulted in alterations in the maximum growth

temperature or kinetic growth parameters at 42 °C. This may indicate different mechanisms for resistance to thermal kill than the ability to grow at high temperatures. Indeed, similar indications have been found in *K. pneumoniae*, as mutation in the heat-resistance mediating gene did not affect the maximum growth temperature (Bojer *et al.*, 2010). Consequently, in experimental designs it may also be important to differentiate between the ability to grow under a certain stress from the ability to resist and survive a harsher stress condition.

In this study, we were able to pinpoint a causative gene carried on a plasmid by comparing two complete genomes and combining the genomic information with traditional molecular experiments. However, a larger sample size would greatly facilitate the prospects of finding completely novel mechanisms conferring stress resistance: a large enough sample size – from hundreds to preferably even thousands – would allow genome-wide association studies (GWAS) methods to be employed that could identify not only complete plasmids or genes but also smaller variants, such as SNPs and indels, associated with certain phenotypes (Lees *et al.*, 2016, Kachroo *et al.*, 2019). It should be noted, however, that even in the case of a proven statistical association, traditional molecular methods are still needed to ascertain true causality (Read and Massey, 2014, Kremer *et al.*, 2017).

The findings in this study raise concern, amongst others, due to the potential cotransfer of plasmid-borne stress resistance-mediating genes together with those conferring resistance to virulence and antibiotics (Bojer *et al.*, 2010, Bojer *et al.*, 2012, Gullberg *et al.*, 2014, Fox *et al.*, 2016). This may provide bacteria with opportunities to populate different niches and to infect new hosts.

6.3 Characteristics of benzalkonium chloride resistance in *L. monocytogenes* (IV)

In this study, 392 *L. monocytogenes* isolates of Finnish and Swiss origin were analysed with respect to BC resistance. Altogether, 11.5% of the strains proved to be BC^r, corresponding with the lower estimates of previous findings in isolates from food-processing environments and foodstuffs (Aase *et al.*, 2000, Mullapudi *et al.*, 2008, Jiang *et al.*, 2016). It should be noted, however, that the definition of BC resistance has differed markedly between studies (Aase *et al.*, 2000, Mereghetti *et al.*, 2000, Xu *et al.*, 2014) due to the often-employed method of using as a threshold the MIC that prevents the growth of the majority of the test strains themselves. However, with large and variable enough sample material, the relative cut-off approach should provide a sufficient basis for selecting the clearly resistant strains. Essentially, these strains would also be the ones most likely to be problematic in food processing premises with regard to the successful disinfection of processing equipment and surfaces. Most worryingly, it has been suggested

that adaptation to BC may also augment antibiotic resistance within bacteria, as was shown within *Pseudomonas* spp. isolates (Tandukar *et al.*, 2013, Kim *et al.*, 2018). In *L. monocytogenes*, adaptation to BC not only enhanced tolerance to subsequent exposure to BC, but also resistance to antimicrobial agents such as cefotaxime, cephalothin and ciprofloxacin (Yu *et al.*, 2018). These outcomes suggest that the use of disinfectants should be well planned and apportioned to avoid the excess use of possibly multi-resistance-selecting agents at futile sites.

No clear correlation between BC resistance and the isolation country or isolation source was found in this study. Considering the serotypes, most of the BC^r strains were of serotype 1/2c, while in total, serotype 1/2a included the highest number of BC^r strains, which may reflect the overrepresentation of 1/2a strains in this study. However, a wide range of BC resistance among different serotypes has previously been reported: Mullapudi *et al.* (2008) found BC^r *L. monocytogenes* strains to be significantly more predominant in serotype 1/2a and 1/2b than within 4b. A parallel distribution of BC^r strains had previously been reported by Mereghetti *et al.* (2000), while others detected no such correlation (Romanova *et al.*, 2002, Soumet *et al.*, 2005, Jiang *et al.*, 2016) and Xu *et al.* (2014), indeed, found an opposite BC resistance pattern among serotypes. It should be noted that the discrepancies might often be explained by the relatively restricted sample sizes or uneven representation of different serotypes among the study strains.

Screening of the known BC resistance-conferring genes *bcrABC* (Elhanafi *et al.*, 2010), *emrE* (Gilmour *et al.*, 2010, Kovacevic *et al.*, 2016) and *qacH* (Müller *et al.*, 2013) revealed fourteen strains lacking these genes altogether, indicating novel mechanisms for BC resistance in *L. monocytogenes*. Furthermore, reserpine screening indicated that in eleven of these strains, at least partially an efflux pump-dependent mechanism, other than those encoded by the known BC resistance-mediating genes, is putatively present. In the three strains both lacking the BC resistance-mediating genes and displaying independence of efflux pumps, a completely novel mechanism of BC resistance is suggested. Indeed, upregulation of peptidoglycan synthesis pathways in the presence of a QAC, benzethonium chloride, was seen in *L. monocytogenes* (Fox *et al.*, 2011), suggesting a potential role of cell-wall modifications in increased tolerance to BC (McDonnell and Russell, 1999). In future research, association study methods would be a noteworthy tool in screening for the potentially completely novel genetic variants putatively conferring BC resistance in *L. monocytogenes*.

7 CONCLUSIONS

1. HKs extensively contribute to stress tolerance in *L. monocytogenes*. YycG and LisK are needed upon an abrupt decline in temperature and cold acclimation, respectively, while LiaS plays a universal role in the tolerance of *L. monocytogenes* to high temperature, acid, alkali, osmotic, ethanol and oxidative stresses. Furthermore, the complete TCSs AgrCA and VirRS play roles under osmotic and ethanol stresses, respectively, and complete YycFG and LisRK under cold conditions. The present studies also revealed indications of potential cross-phosphorylation between noncognate TCS partners of *L. monocytogenes* under ambient stresses.

2. A novel plasmid, pLM58, confers heat resistance in *L. monocytogenes*. Furthermore, the plasmid-borne ATP-dependent protease ClpL contributes to the survival of *L. monocytogenes* at high temperatures, adding to the concern of resistance-mediating genes efficiently spreading by horizontal gene transfer among bacterial populations.

3. Emerging BC resistance is a potential concern in *L. monocytogenes* strains of Finnish and Swiss origin. BC resistance in strains lacking the so far known BC resistance-mediating genes indicates the existence of novel BC resistance mechanisms.

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